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PROVISIONAL APPLICATION COVER SHEET

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Additional inventors are being named on the separately numbered sheet attached hereto.

TITLE OF THE INVENTION (500 characters max)

METHOD OF USING AMMONIA OXIDIZING BACTERIA

CORRESPONDENCE ADDRESS

CUSTOMER NUMBER: 37462

ENCLOSED APPLICATION PARTS (check all that apply)

- Specification Number of Pages 70
- Drawing(s) Number of Sheets 4
- Application Data Sheet, See 37 CFR 1.76
- Return receipt postcard
- Other (specify) _____

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

No

Yes, the name of the U.S. Government Agency and the Government Contract Number are:

METHOD OF PAYMENT (check all that apply)

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Respectfully submitted,

September 26, 2003
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METHODS OF USING AMMONIA OXIDIZING BACTERIA

Field of Invention

- 5 The present invention relates to a composition including ammonia oxidizing bacteria to increase production of nitric oxide and nitric oxide precursors on the surface of a subject and methods of using same to slow the progression of aging and treat and prevent hypertension, hypertrophic organ degeneration, Raynaud's phenomena, fibrotic organ degeneration, allergies, autoimmune sensitization, end stage renal
10 disease, obesity, impotence and cancer with autotrophic ammonia oxidizing bacteria, specifically by administering nitric oxide to a subject.

Background

- 15 Living in an industrialized country has many advantages regarding human health. The causes of death in the developed world tend to be the chronic degenerative diseases of aging, heart disease, kidney failure, Alzheimer's, liver failure, and cancer while the major causes of death in the undeveloped world tend to be acute causes such as infection, starvation and war. However, many people living in third world
20 countries have health profiles that seem "better" than their developed world age matched controls. They have a lower body mass index, lower blood pressure, lower incidence of diabetes, less kidney failure, less heart disease, fewer allergies, less autoimmune disease, less Alzheimer's. The difference is equally apparent even within the same country, between urban and rural dwellers, between rich and poor.
25 Many of the differences are especially apparent in those with dark skin. Adult immigrants, born and raised in undeveloped countries, who move to developed countries typically have better health profiles than do their children born and raised in the developed country.
- 30 Many of the chronic degenerative diseases of the developed world correlate positively with excess body fat. Obesity worsens the prognosis for virtually every chronic disease. Yet not every obese person gets these diseases, and not everyone with these diseases is obese. Some diseases such as cancer, don't seem to have an "obvious" cause, they seem to strike almost at random. In an earlier age, people would have

attributed such diseases to “evil spirits” or “angering the gods.” Now, the “conventional wisdom” is that the “cause” of all of these degenerative diseases is that people do not exercise enough, watch too much TV, eat too many “refined” foods with “too much” fat, sugar, and salt, and are exposed to too many “chemicals”. This is believed to occur in spite of the modern preoccupation with being thin. Changing one’s diet by only 100 calories a day will cause one to gain (or lose) about 10 pounds in a year. In the rural undeveloped world, it would seem unlikely that there is virtually no one who has access to an extra 100 calories a day of food. If anything, obesity should be more common in the undeveloped world, because without refrigeration, excess food is best stored by being eaten and stored as fat. Similarly, is every adult who desires to lose weight, so weak-willed that they cannot reduce their intake by 100 calories a day?

The degenerative diseases of the industrialized world which are exacerbated by obesity are leading causes of death. Many billions have been spent trying to prevent and cure these seemingly disparate disorders, yet the numbers of obese individuals whose health is made worse by their obesity is increasing. A method to prevent these degenerative disorders would have major health implications.

20

Summary

One embodiment of the invention is directed to a method of treating a subject who has developed or is at risk of developing at least one of treating hypertension, hypertrophic organ degeneration, Raynaud’s phenomena, fibrotic organ degeneration, allergies, autoimmune sensitization, end stage renal disease, obesity, impotence and cancer. The method comprises positioning ammonia oxidizing bacteria in close proximity to the subject. In one aspect, the ammonia oxidizing bacteria may be selected from the group consisting of any of *Nitrosomonas*, *Nitrosococcus*, *Nitrosospira*, *Nitrosocystis*, *Nitrosolobus*, *Nitrosovibrio*, and combinations thereof.

In another embodiment, a method for retarding the progression of aging of a subject comprising positioning ammonia oxidizing bacteria in close proximity to the subject is disclosed.

Another embodiment of the invention is directed to augmenting animal growth comprising removing AAOB from the surface of the animal.

Brief Description of the Drawings

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Fig. 1 shows a plot of liver enzymes, alanine transaminase levels (SGPT or ALT) for a single individual both before and during application of AAOB to the scalp and body;

10 Fig. 2 shows the incidence of Alzheimer's Disease verses minimum temperature during the hottest month for a number of cities;

Fig. 3 shows the number of US patents issued on shampoo verses the year of issue;

Fig. 4 shows NO absorption verses biofilm thickness.

Detailed Description

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The present invention relates to a composition including ammonia oxidizing bacteria to increase production of nitric oxide and/or nitric oxide precursors in close proximity to a surface of a subject and methods for slowing the progression of aging and treating and preventing hypertension, hypertrophic organ degeneration, Raynaud's

20 phenomena, fibrotic organ degeneration, allergies, autoimmune sensitization, end stage renal disease, obesity, impotence and cancer with autotrophic ammonia oxidizing bacteria by administering nitric oxide to a subject. "Subject," as used herein, is defined as a human or vertebrate animal including, but not limited to, a dog,

cat, horse, cow, pig, sheep, goat, chicken, primate, e.g., monkey, rat, and mouse. The 25 term "treat" is used herein to mean prevent or retard the onset of a disease or disorder as well as to retard or stop the progression of disease or disorder after its onset.

According to an embodiment of the invention, nitric oxide, a nitric oxide precursor, and/or a nitric oxide releasing compound may be positioned in close proximity to a surface of a subject to slow the progression of aging and treat and prevent

30 hypertension, hypertrophic organ degeneration, Raynaud's phenomena, fibrotic organ degeneration, allergies, autoimmune sensitization, end stage renal disease, obesity, impotence and cancer.

- According to one aspect of the invention, it is appreciated that most chronic degenerative diseases of the modern world, as well as obesity and many cancers may be the natural consequence of the body's natural physiological response to modern bathing practices that wash away a substantial amount of previously unknown
- 5 commensal autotrophic ammonia oxidizing bacteria (AAOB). Accordingly, one aspect of the invention is that these degenerative diseases and obesity may be treated or prevented by applying the AAOB on or in close proximity to a subject. Similarly, another aspect of the invention is that these degenerative diseases may be treated or prevented by not bathing.

10

- More specifically, in one embodiment, applying a composition of an autotrophic ammonia oxidizing bacteria to skin during or after bathing to metabolize urea and other components of perspiration into nitrite and ultimately into Nitric Oxide (NO) results in a natural source of NO. One aspect of the present invention causes topical
- 15 nitric oxide release at or near the surface of the skin where it can diffuse into the skin and have local as well as systemic effects. This nitric oxide can then participate in the normal metabolic pathways by which nitric oxide is utilized by the body. Examples of ammonia oxidizing bacteria include, but are not limited to, *Nitrosomonas*, *Nitrosococcus*, *Nitrosospira*, *Nitrosocystis*, *Nitrosolobus*, *Nitrosovibrio*, and
- 20 combinations thereof, as disclosed in PCT Publication No. WO 03/057380 A2 and PCT Publication No. WO 02/13982 A1, both of which are herein incorporated by reference for all purposes.

- 25 In order to understand the beneficial aspects of these bacteria, it is helpful to understand angiogenesis. All body cells, except those within a few hundred microns of the external air, receive all metabolic oxygen from the blood supply. The oxygen is absorbed by the blood in the lung, is carried by red blood cells as oxygenated hemoglobin to the peripheral tissues, where it is exchanged for carbon dioxide, which
- 30 is carried back and exhaled from the lung. Oxygen must diffuse from the erythrocyte, through the plasma, through the endothelium and through the various tissues until it reaches the mitochondria in the cell which consumes it. The human body contains about 5 liters of blood, so the volume of the circulatory system is small compared to

that of the body. Oxygen is not actively transported. It passively diffuses down a concentration gradient from the air to the erythrocyte, from the erythrocyte to the cell, and from the cell to cytochrome oxidase where it is consumed. The concentration of oxygen at the site of consumption is the lowest in the body, and the O₂ flux is determined by the diffusion resistance and the concentration gradient. Achieving sufficient oxygen supply to all the peripheral tissues requires exquisite control of capillary size and location. If the spacing between capillaries were increased, achieving the same flux of oxygen would require a larger concentration difference and hence a lower O₂ concentration at cytochrome oxidase. With more cells between capillaries, the O₂ demand would be greater. If the spacing between capillaries were decreased, there would be less space available for the cells that perform the metabolic function of the organ.

In one aspect of the invention, it is appreciated that NO from autotrophic ammonia oxidizing bacteria (AAOB) is readily absorbed by the outer skin and converted into S-nitrosothiols since the outer skin is free from hemoglobin. The external skin receives all of its oxygen from the external air.¹ This is readily apparent, because the external skin can be seen to be essentially erythrocyte free. There is circulation of plasma through these layers because they are living and do require the other nutrients in blood, just not the oxygen. S-nitrosothiols formed are stable, can diffuse throughout the body, and constitute a volume source of authentic NO and a source of NO to transnitrosate protein thiols.

In another aspect of the invention, it is appreciated that capillary rarefaction may be one of the first indications of insufficient levels of NO. The human body grows from a single cell, and damaged vasculature is efficiently healed in all tissues. The regulation of angiogenesis and vascular remodeling is the subject of intense research, and a number of factors are well understood. .

Sparse capillaries, or capillary rarefaction, is commonly seen in people with essential hypertension.² Capillary rarefaction is seen in people “at risk” for hypertension before they develop it.³ There is as yet no good explanation for the cause of capillary rarefaction, but there is both a reduced density of capillaries, and reduced recruitment

of capillaries in response to increased local blood demand.⁴ It is easy to see how capillary rarefaction could lead to hypertension. The metabolic demand of a volume of tissue does not go down as the capillary density goes down, so the volumetric blood flow through the sparser network of capillaries must stay the same. With the 5 same volumetric flow but with a reduced cross section available for flow, the pressure drop must increase. It is observed that microvascular rarefaction does lead to increased pressure drop.⁵ With an increased path length for O₂ diffusion from the capillary to the cells farthest from the capillary, the O₂ concentration at those farthest cells must decrease to maintain the same O₂ flux.⁶ In this last reference they show 10 that in addition to greater hypoxia, the heterogeneity of oxic/hypoxic regions is much greater under conditions of capillary rarefaction, and that fluctuation between oxic/hypoxic states increases.

In another aspect of the invention it, is appreciated that it is not merely the 15 concentration of O₂ that affects capillary rarefaction, but also O₂ chemical potential. The O₂ chemical potential is directly proportional to O₂ partial pressure and is proportional to the concentration dissolved in the erythrocyte free plasma and in the extracellular fluid. The chemical potential of O₂ in an erythrocyte is equal to that of the plasma in equilibrium with it. O₂ diffuses from the capillary through the 20 hemoglobin-free tissues to reach the cells that are remote from a capillary.

A great many conditions are associated with the capillary density becoming sparser. Hypertension has been mentioned earlier, and sparse capillaries are also seen in the children of people with essential hypertension,⁷ and also in people with diabetes.^{8 9} 25 Significant complications of diabetes are hypertension, diabetic nephropathy, diabetic retinopathy, and diabetic neuropathy. The last two conditions are characterized by a reduction in blood flow to the affected areas prior to observed symptoms.¹⁰ Reduced capillary density is associated with obesity, and simple weight loss increases capillary density.¹¹ In primary Raynaud's phenomena (PRP), the nailfold capillaries are 30 sparser (slightly) than in normal controls, and more abundant than in patients that have progressed to systemic sclerosis (SSc).¹² They found that the capillary density decreased from 35 loops/mm² (normal controls) to 33 (PRP), to 17 (SSc). The

average distance between capillary limbs was 18 μ , 18 μ , and 30 μ for controls, PRP and SSc.

- In another aspect of the invention, it is appreciated that the mechanism that the body normally uses to sense "hypoxia" may affect the body's system that regulates capillary density. According to this aspect of the invention, a significant component of "hypoxia" is sensed, not by a decrease in O₂ levels, but rather by an increase in NO levels. Lowering of basal NO levels interferes with this "hypoxia" sensing, and so affects many bodily functions regulated through "hypoxia." For Example, anemia is commonly defined as "not enough hemoglobin," and one consequence of not enough hemoglobin is "hypoxia", which is defined as "not enough oxygen." According to one aspect of the invention, these common definitions do not account for the nitric oxide mediated aspects of both conditions.
- At rest, acute isovolemic anemia is well tolerated. A 2/3 reduction in hematocrit has minimal effect on venous return PvO₂, indicating no reduction in either O₂ tension or delivery¹³ throughout the entire body. At 50% reduction (from 140 to 70g Hb/L), the average PvO₂ (over 32 subjects) declined from about 77% to about 74% (of saturation). The reduction in O₂ capacity of the blood is compensated for by vasodilatation and tachycardia with the heart rate increasing from 63 to 85 bpm. That the compensation is effective is readily apparent, however, the mechanism is not. A typical explanation is that "hypoxia" sensors detected "hypoxia" and compensated with vasodilatation and tachycardia. However, there was no "hypoxia" to detect. There was a slight decrease in blood lactate (a marker for anaerobic respiration) from 0.77 to 0.62 mM/L indicating less anaerobic respiration and less "hypoxia." The 3% reduction in venous return PvO₂ is the same level of "hypoxia" one would get by ascending 300 meters in altitude (which from personal experience does not produce tachycardia). With the O₂ concentration in the venous return staying the same, and the O₂ consumption staying the same, there is no place in the body where there is a reduction in O₂ concentration. Compensation during isovolemic anemia may not occur because of O₂ sensing.

“Hypoxia” from other causes does not have the same effect on cardiac output. When a portion of a dog’s normal erythrocytes are replaced with erythrocytes that are fully oxidized to metHb, “hypoxic” compensation is minimal¹⁴. While maintaining the same hematocrit Hct (43%) and substituting (0, 26, 47%) fully metHb erythrocytes, 5 the cardiac output (CO) declined (178, 171, 156 mL/m/kg) while the arterial PaO₂ (93, 87, 84 mmHg) and PvO₂ (55, 46, 38) also declined. In contrast, when acute isovolemic anemia (Hct 40, 30, 22) was induced using plasma, compensation was much better, CO (155, 177, 187), PaO₂ (87, 88, 91), and PvO₂ (51, 47, 42). When anemia was induced using dextran solution (Hct 41, 25, 15) cardiac output (143, 195, 10 243), PaO₂ (89, 92, 93), PvO₂ (56, 56, 51) compensation was better still.

As part of their experiments with the metHb tests, a final dilution was done with dextran to lower the Hct to 26% while still maintaining 47% metHb. Compensation was much improved with CO (263 mL/m/kg), PaO₂ (86 mmHg), and PvO₂ (41 15 mmHg) all were increased, despite lower Hct, greater O₂, and less “hypoxia.” The compensatory mechanisms to deal with this “hypoxia” may not be due to reduced O₂ levels because the O₂ levels were not reduced, in fact, the O₂ levels were increased.

Pulmonary gas exchange efficiency improves during isovolemic anemia, and exhaled 20 NO increases as Hct decreases (in rabbits)¹⁵. As Hct was decreased by dilution with hydroxyethyl starch (30, 23, 17, 11 %), cardiac output rose (0.52, 0.60, 0.70, 0.76 L/min), and exhaled NO levels rose (30, 34, 38, 43 nL/min).

Maximum O₂ consumption (VO_{2max}) is reduced at high altitude, and this reduced 25 VO_{2max} is not restored by acclimatization.¹⁶ VO_{2max} is decreased when hematocrit is decreased in spite of no difference in PaO₂ or PvO₂¹⁷. In this last reference, Koskolou et al.’s data clearly show a 17% reduction in maximum work, with Hb change (154.4 to 123.3 g/L) a PaO₂ change (119.2 to 115.1 mmHg) and a PvO₂ change (23.6 to 23.0 mmHg). Koskolou et al. do not have an explanation for the 30 inability of the trained muscle to “extract” the oxygen which is being delivered by the blood, or the inability of the heart to deliver more blood despite reserve cardiac capacity. This behavior may be explained by the interaction of NO with heme

proteins and the competitive inhibition of cytochrome oxidase by NO causing reduced VO_{2max}.

Horses when treated with the NOS inhibitor L-NAME showed an accelerated increase
5 in VO₂ and a lower “oxygen debt”, but also a slightly lower VCO_{2max}¹⁸. The
accelerated VO₂ was attributed to reduced NO inhibition of mitochondrial respiration,
and the slightly reduced VCO_{2max} (62.5, 61.0 L/min) to the reduced cardiac output
(which was reduced 12% due to vasoconstriction) observed in the L-NAME group.
The increased VO_{2max} observed with increases in Hct is as in “blood doping” is
10 likely due to decreased NO as well. These examples are all consistent with NO
inhibition of mitochondrial respiration and that inhibition being modulated by changes
in hematocrit.

Hb is well known to remove NO from solution with kinetics that are first order in both
15 Hb and NO¹⁹. At steady state, the NO production rate will be constant, and the
production rate equals the destruction rate (no accumulation). A sudden drop in
hematocrit by 50% will result in an increase in NO concentration because the
production rate would continue to equal the destruction rate and as the destruction
rate is first order in both NO and Hb it is their product that remains constant. The
20 reaction between NO and Hb is so fast, that the new NO concentration will be reached
virtually as soon as the blood and the diluent mix and pass by a vessel wall.

Thus the vasodilatation that is observed in acute isovolemic anemia may be due to
the increased NO concentration at the vessel wall. NO mediates dilatation of vessels
25 in response to shear stress and other factors. No change in levels of NO metabolites
would be observed, because the production rate of NO is unchanged and continues to
equal the destruction rate. The observation of no “hypoxic” compensation with
metHb substitution can be understood because metHb binds NO just as Hb does, so
there is no NO concentration increase with metHb substitution as there is with Hb
30 withdrawal.

Many details of NO chemistry while well known are not universally well appreciated.
The ligands O₂, CO, H₂S and HCN, along with NO, all bind to heme and may at

- times be significant in human physiology. The activity of all proteins containing heme (and there are many) will therefore be affected by the concentrations of all of these species. Sometimes, one or several can be ignored, but the circumstances under which a potential activating species can be ignored must be well considered because
- 5 the binding constants for NO, CO, H₂S, and HCN are many orders of magnitude greater than that of the most abundant ligand, O₂. The various heme containing proteins don't "sense" any of these ligands independently; they only "sense" relative concentrations of all the ligands.
- 10 The behavior of NO and NOS enzymes in the body are complex. The gene for one isoform nNOS is, "the most structurally diverse human gene described to date in terms of promoter usage"²⁰. NO is difficult to measure, is active at very low levels, is labile, reactive, and diffuses rapidly, so concentrations change rapidly in time and space. It is active at many diverse sites where it serves diverse signaling and
- 15 regulatory functions through multiple mechanisms. It is responsible for regulation of vascular tone through cGMP mediated relaxation of smooth muscle. It is responsible for regulation of O₂ consumption by cytochrome oxidase by competitively inhibiting O₂ binding. It is responsible for inhibition of proteases, including caspases, by S-nitrosylation of cysteine residues and induces expression of matrix
- 20 metalloproteinases. NO is a major component of the immune reaction, and is produced in large quantities by iNOS in response to infection. It should also be recognized that the length scale over which NO gradients are important extends to individual cells. It should also be recognized that not all "NO effects" are mediated through "free NO". S-nitrosothiols can transnitrosate protein thiol groups without
- 25 free NO ever being present. The state of the art in NO measurement does not allow measurement on the time, distance and concentration scales that are known to be important. With this level of complexity and experimental difficulty, it is not surprising that the details of how NO interacts with hemoglobin (which is perhaps the best understood human protein) are not agreed upon by those most knowledgeable in
- 30 the field.

It is known that Nitric oxide plays a role in many metabolic pathways. It has been suggested that a basal level of NO exerts a tonal inhibitory response, and that

reduction of this basal level leads to a dis-inhibition of those pathways.²¹ NO has been shown to inhibit basal sympathetic tone and attenuate excitatory reflexes.²²

One function of NO is to regulate O₂ consumption by cytochrome oxidase by binding 5 to cytochrome oxidase and competitively inhibiting the binding of O₂. Inhibition of O₂ is advantageous because the concentration of O₂ at each mitochondria in every cell cannot be well controlled. As O₂ is consumed, the O₂ level drops, more NO binds, and the inhibition increases, slowing the consumption of the remaining O₂. Without this inhibition, the mitochondria closest to the O₂ source would consume 10 more, and those far away would get little or no O₂. For some tissues, such as heart muscle, the O₂ consumption can change by a factor of more than 10 between basal and peak metabolic activity. To achieve this O₂ flux, the gradient must increase because the capillary spacing does not change with O₂ consumption (although there is some increased recruitment of capillaries which were otherwise empty). Decreasing 15 NO concentrations increase the rate of O₂ consumption by mitochondria by removing the inhibition that NO produces.

The inhibition of cytochrome oxidase by NO may depend on the relative concentrations of both NO and O₂. Thus the reduction of VO_{2max} during hypobaric 20 hypoxia may be due to less O₂ relative to the same NO while the reduction of VO_{2max} during isovolemic anemia may be due to increased NO relative to the same O₂. The increase in exhaled NO during isovolemic anemia is due to less trapping and destruction in the lung of NO produced in nasal passages. The reduced O₂ delivery to muscle during isovolemic anemia is due to greater NO levels. With greater NO 25 concentration, the operating point of the mitochondria is shifted to a higher O₂ concentration. The concentration of O₂ at the mitochondria is actually increased during isovolemic anemia due to greater inhibition by NO. With higher concentration at the O₂ sink, the concentration gradient is less and so the O₂ flux is less. The reduction in blood lactate during isovolemic anemia demonstrates that the 30 mitochondria may actually be less hypoxic, so anaerobic glycolysis is less. The adverse consequence of decreased NO levels leading to increased anaerobic glycolysis will be discussed later.

Reductions in VO_{2max} can be observed in hypobaric hypoxia and isovolemic anemia, and VO_{2max} increases are observed with L-NAME inhibition. This demonstrates that the NO concentration at the mitochondria is coupled to the hemoglobin concentration in the blood by destruction of NO by hemoglobin and to NO production by NOS.

- 5 NO binds to the heme of many proteins. Because most of the body's iron is in hemoglobin, the concentration of heme in the blood is much higher than in any other tissue, so the binding of NO by heme will be most rapid there and the blood is
- 10 considered to be the major sink of NO. A major source of NO is the endothelium where eNOS is constitutively expressed. With the source of NO and the sink of NO so close together, the NO concentration at regions remote from the source and sink will be sensitively dependant on the details of the source-sink interactions. There are other sources of NO as well. Blood and plasma contains a number of S-nitrosothiols
- 15 of which the major one is S-NO-albumin.²³ NO can be cleaved from S-nitrosothiols with light, and by various enzymes including xanthine oxidase,²⁴ copper ions and copper containing enzymes including Cu,Zn SOD.²⁵ Many of the metabolic functions of NO do not require liberation of free NO. When a cysteine in the active region of a protein is S-nitrosylated, the activity of the protein is affected.²⁶ Transfer of NO from one S-nitrosothiol to another is termed transnitrosation,²⁷ and is catalyzed by a number of enzymes including protein disulfide isomerase.²⁸ Many of the metabolic effects of NO are known to be mediated through S-nitrosothiols, for example S-nitrosothiols mediate the ventilatory response to hypoxia.²⁹
- 20
- 25 In the example of a 50% reduction in hematocrit, the NO concentration at the capillary wall will increase to match the prior destruction rate, and may double. NO will also passively diffuse throughout the body, and with the major sink being the hemoglobin in the blood, the concentrations elsewhere will increase too. It should be noted, that with the sink being the hemoglobin, the minimum NO concentration
- 30 occurs at the site of consumption, the hemoglobin in the blood. Thus there will naturally be a gradient of NO concentration that is the reverse of the O₂ gradient, provided there is a source of NO in the peripheral tissues. Although NOS is

expressed in many tissues, such a source has not been reported (probably largely due to the experimental difficulty of measuring NO gradients between capillaries).

- In one aspect of the invention, it is appreciated that one component of this volume
- 5 source of NO is low molecular weight S-nitrosothiols produced in the erythrocyte free skin from NO produced on the external skin by autotrophic ammonia oxidizing bacteria. These low molecular weight S-nitrosothiols are stable for long periods, and can diffuse and circulate freely in the plasma. Various enzymes can cleave the NO from various S-nitrosothiols liberating NO at the enzyme site. It is the loss of this
- 10 volume source of NO from AAOB on the skin that leads to disruptions in normal physiology. The advantage to the body of using S-nitrosothiols to generate NO far from a capillary is that O₂ is not required for NO production from S-nitrosothiols. Production of NO from nitric oxide synthase (NOS) does require O₂. With a sufficient background of S-nitrosothiols, NO can be generated even in anoxic regions.
- 15 Free NO is not needed either since NO only exerts effects when attached to another molecule, such as the thiol of a cysteine residue or the iron in a heme, so the effects of NO can be mediated by transnitrosation reactions even in the absence of free NO provided that S-nitrosothiols and transnitrosation enzymes are present.
- 20 In another embodiment of the invention, it is appreciated that in the absence of overt anoxia, elevated NO may be a more effective "hypoxia" signal to regulate hematocrit and other "hypoxia" mediated factors, than depressed O₂. Since the "normal" hematocrit set point is determined in the absence of overt hypoxia, the "normal" Hct setpoint may be determined by NO and not O₂ levels, or more precisely, by the ratio of NO to O₂ (NO/O₂). The "hypoxia" signal need not be linear with NO/O₂, but the "hypoxia" signal may increase with increased NO and may increase with decreased O₂. Each may have an effect on the "hypoxia" signal, but not necessarily an equal effect.
- 25 Similarly, the vascular remodeling that normally occurs continuously and in the absence of overt anoxia must also be regulated through a "hypoxia" signal that also occurs continuously and in the absence of overt anoxia. When blood flow to a capillary bed is reduced, O₂ delivery to portions of the tissue served by that bed is

reduced. This results in the heterogeneous appearance of hypoxia, with the cells farthest (in the sense of O₂ diffusion resistance) from the capillaries experiencing hypoxia first. This has been observed *in vitro*, where perfused rat hearts were infused with a Pd porphine which has its fluorescence quenched by O₂, and the fluorescence of the Pd porphine and the fluorescence of NADH (a measure of mitochondria deoxygenation) were observed during normoxic and hypoxic perfusion.³⁰ During the transition from anoxic to normoxic conditions, the regions that had less O₂ matched those that had greater NADH, and the length scale of the heterogeneity of those regions matched that of the capillaries. The literature demonstrates that “hypoxia” is a local effect, it is heterogeneous at the capillary level, that heterogeneity is due to capillary spacing, and that “hypoxia” due to stopped flow has the same heterogeneity as “hypoxia” due to anoxic fluid at high flow. The greatest heterogeneity was observed during recovery from anoxia. It should also be noted that in the absence of sufficient NO, the activity of cytochrome oxidase for O₂ is greater, that is the activity at a given O₂ concentration is greater. Thus cells in close proximity to capillaries will consume more O₂ leaving even less for cells far from a capillary. Insufficient NO will exacerbate the degree of heterogeneity of hypoxia, and will therefore increase the number of transitions between hypoxic and oxic conditions. The production of superoxide is greatest during reoxygenation following hypoxia. The mitochondria respiration chain becomes fully reduced, and O₂ captures the electron before it can be shuttled to cytochrome oxidase. With a reduced NO level, the operating point of the mitochondria is shifted to a lower O₂ concentration. This means that there is less “capacitance” due to O₂ stored in the tissues. More superoxide gets produced, and because superoxide destroys NO with diffusion limited kinetics, more superoxide means even less NO. This destruction of NO by superoxide caused by local hypoxia may exacerbate conditions of insufficient perfusion.

The O₂ partial pressure of the blood is normally quite constant and very well regulated. In order to regulate the spacing of capillaries, the body must measure the diffusion resistance of O₂ to that site and generate capillaries where the O₂ diffusion resistance is too high, and ablate capillaries where the resistance is too low. The oxygen demand of tissues fluctuates with their metabolic activity, and the “normal” capillary spacing must be sufficient for “normal” metabolic demand (plus some

reserve). The simplest way that O₂ diffusion resistance can be determined and hence regulated is to decrease supply at constant demand. The alternative, increasing demand at constant supply, would require a method to dissipate the metabolic heat that would be liberated, which is not observed. Since the demand must exceed the supply, a “hypoxic” state must be induced, at which time normal functionality must be compromised (otherwise it wouldn’t be hypoxia). Decreasing the O₂ concentration or flow rate of blood, while maintaining basal metabolic load, would induce a state of hypoxia and so allow cells to determine the diffusion resistance of O₂. Since metabolic functionality is necessarily compromised, a preferred time to do this would be when metabolic demand is at a minimum, when the organism is not moving or needing to evade predators, such as during sleep. Inducing hypoxia at the lowest metabolic rate also results in the longest time constant, which minimizes the chance of overshoot and hypoxic damage.

15 Erythropoiesis is mediated in part through erythropoietin (EPO), which is produced primarily by the kidney in response to “hypoxic” stimuli,³¹ including hypobaric hypoxia, isovolemic anemia, cobalt chloride, and deferoxamine. Many of the effects of “hypoxia” are mediated through hypoxia-inducible factor (HIF-1a) which activates transcription of dozens of genes including the EPO gene³². Complex behavior of HIF-1a in response to NO exposure has been demonstrated using authentic NO, NO donors and also transfected cells expressing iNOS as NO sources.³³ Sandau et al. found that lower NO levels induced a more rapid response and produced more HIF-1a than did higher levels. The only NO donor tested which did not induce HIF-1a was sodium nitroprusside which also releases cyanide. They also determined that the induction of HIF-1 a was not mediated through cGMP. Angiogenesis is mediated in part through VEGF, which is induced by HIF-1a which is induced by NO³⁴. The angiogenesis that accompanies normal wound healing is produced in part by elevated VEGF which is induced by increased nitric oxide³⁵.

20 Thus, when hypoxia is not accompanied by sufficient NO, a lower level of oxygen for a longer period of time is required to elicit induction of HIF-1a and VEGF. It should be remembered that with low NO levels, mitochondrial consumption of O₂ is faster,

so the O₂ level will drop faster and farther and for a longer period of time than with high NO.

According to another embodiment of the invention, it is appreciated that accelerated turnover of organ cells by hypoxia induced by capillary rarefaction may be a factor in the accelerated aging that is observed in the chronic degenerative diseases. The body controls spacing between capillaries so as to match the local O₂ demand with the local blood supply. To do this, it induces a state of "hypoxia" and, through HIF-1a and VEGF, initiates angiogenesis where needed. To ensure that the capillaries are not too close, there may also be a signal indicating an absence of nearby "hypoxia" which may lead to capillary ablation, through endothelial cell apoptosis. This ablation may be mediated through the absence of VEGF (or other endothelial cell survival factors) diffusing from "hypoxic" cells nearby. VEGF deprivation does induce apoptosis in endothelial cells.³⁶ Insufficient VEGF, due to low basal NO, from cells that have insufficient O₂ but which don't have the NO/O₂ ratio to initiate Hif-1a prevents new capillaries from being formed and ablates already formed nearby capillaries by depriving them of VEGF. Thus low basal NO may induce a state of chronic insufficient O₂ in that population of cells farthest from the capillaries, and may increase the average spacing between capillaries. The number of cells that may be affected at any one time is small, and may occur in isolated regions with lengths scales less than the capillary spacing. Moreover, cells may be affected only one at a time. Such an isolated hypoxic cell would be difficult to detect. When such a cell dies through apoptosis or necrosis, the resulting inflammation would also be difficult to detect. Over time, affected cells would die and be cleared, the geometry of the capillary structure would collapse, new cells would move into the hypoxic zone, more capillaries would ablate, and over many years, many of the cells of an organ could be affected. If surviving cells divide to replace the ones that die, the cycle of cell death and cell replacement could occur many times, and over many years the number of so affected cells could exceed the total in the organ, perhaps even by many fold. With each cell division, the telomeres in the cell become shorter, and when the telomeres become too short, the cell can no longer divide.

According to an embodiment of the invention, it is appreciated that capillary rarefaction can then be seen as the consequence of too little NO at cells remote from a capillary. Without enough NO, the cells may not produce the signal to initiate angiogenesis. In spite of chronic low O₂, without enough NO there is no "hypoxic" signal to initiate angiogenesis. However, cells require O₂ for oxidative phosphorylation to supply the ATP and other species needed to perform the various metabolic functions. With inadequate O₂, cell function will be degraded. It should be noted that in the absence of sufficient NO, the O₂ gradient (dO_2/dx) is steeper due to the lack of inhibition of cytochrome oxidase at low O₂. Thus cells that are beyond the NO/O₂ threshold for inducing angiogenesis may experience greater hypoxia induced dysfunction. Some cells can generate ATP through anaerobic glycolysis. However, anaerobic glycolysis consumes 19 times more glucose than does aerobic glycolysis per unit of ATP generated. If even a few cells are producing ATP through anaerobic glycolysis, the local glucose concentration may become depleted. The effect of this localized depletion in glucose levels due to hypoxia will be apparent later.

Reliance on anaerobic glycolysis has another effect, the generation of NADH, or reducing equivalents. These reducing equivalents cannot be oxidized because there is insufficient O₂. One way for the cell to "dispose" of them is to use them in the synthesis of lipids. This may be one source of the liver lipids observed in non-alcoholic steatohepatitis. Just as the metabolism of alcohol by the liver produces "excess" reducing equivalents which lead to fatty liver, so to may anaerobic glycolysis due to chronic diffuse hypoxia from capillary rarefaction.

When cells are hypoxic, or when they alternate between oxic and hypoxic states, the production of superoxide is increased. This superoxide further decreases NO levels because NO and superoxide react with diffusion limited kinetics, and will exacerbate any effects of low NO. This may be what brings on the NO crisis and the constricted capillaries of Raynaud's phenomena. When capillaries become rarefacted, the tissue is especially sensitive to any hypoxic insult, to any change that decreases the perfusion of the volume of tissue, such as cold. When this happens, the tissue becomes hypoxic, superoxide is produced, NO is destroyed, capillaries become more

constricted due to reduced vasodilatation which leads to further hypoxia, further superoxide and further constriction. The hypoxia exacerbates the low NO and vice versa. It is a case of positive feedback. One solution is to stop the capillary rarefaction in the first place. When NO is destroyed with superoxide, peroxynitrite is formed. Peroxynitrite is a strong oxidant which affects a number of enzymes. An enzyme that is affected is eNOS. eNOS synthesizes NO from L-arginine, O₂, NADPH, and tetrahydrobiopterin.³⁷ Electrons are shuttled from NADPH, through calmodulin and onto the eNOS dimer. When the eNOS dimer is exposed to peroxynitrite, the zinc thiolate complex is destabilized, and eNOS becomes “uncoupled”. It produces superoxide instead of NO.³⁸

In another aspect of the invention it, is appreciated that peroxynitrite injury may not be a case of too much NO, but may be a case of too little. Many of the experimental results showing increased damage due to increased NO, may be artifacts of the experimental techniques used. Most NO donors used in such experiments release NO indiscriminately. It is not surprising that releasing a compound as reactive as NO indiscriminately causes problems. Similarly, many of the NOS inhibitors not only inhibit NO production, they also inhibit superoxide production by NOS. Thus a “protective” effect of a NOS inhibitor on ischemic injury, doesn’t necessarily demonstrate that the injury is a result of NO.

Even if only one cell becomes hypoxic, around that cell the resulting superoxide will destroy NO and the cell and cells in the vicinity will become further depleted in NO. With less NO, the signals of HIF-1 α and VEGF will be attenuated, and capillary rarefaction may progress.

Reliance solely on O₂ levels for control of capillary spacing would be problematic in tissues where O₂ levels do not reflect capillary spacing, such as in the gas exchange regions of the lung.

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Cancer

According to another embodiment of the invention, it is appreciated that the presence of NO during hypoxia may prevent cells from dividing while under hypoxic stress, when cells are at greater risk for errors in copying DNA. One cell function is the regulation of the cell cycle. This is the regulatory program which controls how and 5 when the cell replicates DNA, assembles it into duplicate chromosomes, and divides. The regulation of the cell cycle is extremely complex, and is not fully understood.³⁹ However, it is known that there are many points along the path of the cell cycle where the cycle can be arrested and division halted until conditions for doing so have improved. The p53 tumor suppressor protein is a key protein in the regulation of the 10 cell cycle, and it serves to initiate both cell arrest and apoptosis from diverse cell stress signals including DNA damage and p53 is mutated in over half of human cancers.⁴⁰ Hypoxia does initiate accumulation of p53, and while hypoxia is important in regulating the cell cycle,⁴¹ hypoxia alone fails to induce the down stream expression of p53 mRNA effector proteins and so fails to cause arrest of the cell 15 cycle.⁴² Hypoxic induction of cell arrest requires hypoxia-inducing factor-1 (HIF-1a)⁴³ and NO is one of the main stimuli for HIF-1a.⁴⁴ In contrast, NO does cause the accumulation of transcriptionally active p53 and does cause arrest of the cell cycle and does cause apoptosis.^{45 46 47} Hypoxia in tumors during cell division increases genetic instability, including increased mutations, deletions and transversions.⁴⁸

20 Hypoxia in tumors selects for tumor cells that are resistant to hypoxia mediated apoptosis.⁴⁹ If an error is introduced in the p53 gene (as has occurred in more than half of all cancers) then that cell (and all daughter cells) no longer has one of the main tumor suppressor genes which prevent cancers from growing uncontrollably. Many tumor cells are quite resistant to hypoxia, hypoxia confers resistance to both

25 chemotherapy drugs and radiation, and many tumors have hypoxic regions.⁵⁰ Tumor invasiveness is increased by hypoxia, and that increase is blocked by compounds that release NO.⁵¹ In this last reference, they also note that the various NOS enzymes use O₂ to generate NO, and so will produce less NO under conditions of hypoxia, exactly the time when more NO is needed. Hypoxia induces the production of VEGF and so

30 reduces apoptosis due to serum deprivation.⁵² There are many growth factors in serum, only some of which have been characterized. One wonders if the increase in insulin (which is also a growth factor for endothelial cells) in type 2 diabetes might be compensatory, to reduce apoptosis of the vasculature due to low basal NO levels.

Administering L-arginine to type 2 diabetics increases insulin sensitivity and increases forearm blood flow⁵³ indicating that reduced basal NO levels are characteristic of type 2 diabetes. The total incidence of cancer, as well as cancers of the breast, liver, kidney, pancreas, colon, brain, and others are all elevated in patients 5 diagnosed with diabetes.⁵⁴

In another aspect of the invention, it is appreciated that early menarche and increased height are markers for increased basal metabolism due to low basal NO. In breast cancer, it is well known that factors that increase risk are early menarche, never being 10 pregnant, never breast feeding, living in a developed region (for example the age-corrected incidence for ethnic Chinese living in Los Angeles is 48.7 per 100,000 while for Chinese living in Shanghai it is 21.2; for ethnic Japanese in L.A. it is 72.2, in Osaka it is 21.9),⁵⁵ living in a urban area,⁵⁶ being tall. Factors that do not seem to affect incidence of breast cancer include PCB or DDT exposure⁵⁷ suggesting that 15 exposure to "chemicals" is not the main factor. I suggest that it is the vascular proliferation and increased capillary density that accompanies pregnancy⁵⁸ and lactation⁵⁹ that provides the protective effects. It has been suggested that the increased exposure to estrogen "hormones" which accompanies early menarche is causal. However, while many breast tumors are estrogen dependant, it is not clear 20 how estrogen would induce the genetic abnormalities that lead to cancer initiation. Pregnancy induces many growth factors, it would seem unlikely that the many growth factors of pregnancy are some how "protective", but the few growth factors of early menarche are "causal". The urban/rural and developed/undeveloped effects may be due to AAOB and their effect on basal NO levels. Many of the known protective 25 factors are consistent with greater capillary density and many of the known risk factors are consistent with decreased capillary density. That the incidence of breast cancer in the developed world is in places more than twice that of the undeveloped World implies that most developed World cancers are caused by the environmental changes accompanying development.

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Migration studies have shown that the breast cancer incidence of migrants initially matches that of location of origin, and over time shifts to match that of the area migrated to. However, the time constant for this shift is on the order of decades.⁶⁰

In another aspect of the invention, it is appreciated that increased sodium intake may increase metabolic load on the kidney and increase sensitivity to ischemic insults, thereby accelerating the progression of low NO induced capillary rarefaction.

- 5 Increased cell division while under hypoxic stress will lead to increased mutations and increase the likelihood of a cancerous transformation. It should be recognized that under conditions of chronic low NO, after capillaries have become rarefacted, the cells farthest from the capillaries are always in a chronic state of hypoxic stress and so are especially sensitive to insults that drive them over the edge and into apoptosis or
- 10 necrosis or genetic instability. Any insult that increases metabolic load will increase the local hypoxia and increase the rate at which they die or mutate. In the kidney, a major metabolic load is due to sodium resorption. Increased sodium will increase metabolic load on the kidney and increase sensitivity to ischemic insults and accelerate the progression of low NO induced capillary rarefaction. This may explain
- 15 why a high salt diet exacerbates hypertension and kidney damage. In the liver, alcohol metabolism can displace up to 90% of other metabolic substrates.⁶¹ Stressing cells in the liver with alcohol would be expected to worsen their response to hypoxic stress. Hypertrophic hearts are especially vulnerable to hypoxia.⁶² Thus many of the recognized risk factors for degenerative diseases are factors that may be well tolerated
- 20 in patients with normal capillary density, but may exacerbate the metabolic deficiency of any tissue with refracted capillaries.

- In another aspect of the invention, it is appreciated that preventing the necrotic death of cells by preventing the capillary rarefaction that leads to their hypoxic death may prevent autoimmune disorders. When cells are exposed to chronic hypoxia, the production of reactive oxygen species (ROS) is increased, and there is increased damage to the cells metabolic machinery and ultimately to the cells DNA. Decreased metabolic capacity will decrease capacity for repair of damage due to ROS and due to exogenous carcinogen exposure. Over time, the damage accumulates and will
- 25 ultimately result in one of 3 events. The cell will undergo deletion of cancer preventing genes and the cell will become cancerous, the cell will die through necrosis, or the cell will die through apoptosis. When cells die, either through necrosis or apoptosis, the cell debris must be cleared from the site. Dead cells are

phagocytosed by immune cells, usually dendritic cells.⁶³ When dendritic cells phagocytose a body, it is digested by various proteolytic enzymes into antigenic fragments, and then these antigens are attached to the major histocompatibility complex (MHC1, MHC2) and the antigen-MHC complex is moved to the surface of the cell where it can interact with T cells and activate the T cells in various ways. Any cell injury releases adjuvants which stimulate the immune system in various ways.⁶⁴ In general, cells that undergo necrosis stimulate a greater immune response than cells that undergo apoptosis.⁶⁵ Chronic exposure of dendritic cells to dead and dying cells is therefore likely to lead to autoimmune disorders. Chronic inflammation is well known to increase cancer incidence.

According to another aspect, it is appreciated that the generalized shrinkage of organs that occurs with age may result from the gradual apoptotic loss of cells due to capillary rarefaction. When cells die through necrosis, they induce inflammation and the cell debris must be phagocytosed for disposal. When necrotic tissue is phagocytosed by dendritic cells the dendritic cells mature and express antigens derived from the necrotic tissue and the major histocompatibility complex resulting in the induction of immunostimulatory CD4+ and CD8+ T cells.⁶⁶ Significant quantities of necrotic tissue (one cell at a time) could very well prime the immune system for autoimmune diseases. In primary Raynaud's phenomena (PRP), the nailfold capillaries are sparser (slightly) than in normal controls, and more abundant than in patients that have progressed to systemic sclerosis (SSc).⁶⁷ They found that the capillary density decreased from 35 loops/mm² (normal controls) to 33 (PRP), to 17 (SSc). The average distance between capillary limbs was 18μ, 18μ, and 30μ for controls, PRP and SSc. Even if only a few cells between each capillary were damaged due to hypoxia at any one time, that damage would accumulate, and eventually, those cells would necrose and be phagocytosed. With so many opportunities for autoimmune sensitization, it would seem only a matter of time before autoimmune sensitization occurred. If the stressed cells are removed through apoptosis, there might be no sign on autopsy that they were ever there. The generalized shrinkage of organs that occurs with age might result from the gradual apoptotic loss of cells due to capillary rarefaction.

Any organ that experiences capillary rarefaction is a candidate for autoimmune sensitization. The progression from PRP to SSc and autoimmune sensitization is simply a reflection of greater capillary rarefaction and increased opportunities for autoimmune sensitization. Similarly, other autoimmune disorders are due to chronic inflammation induced by capillary rarefaction.

In another aspect of the invention, it is appreciated that low basal NO leads to fibrotic hypertrophy. Once a dead cell has been cleared, a new cell cannot easily take its place, because there is insufficient O₂ to support it. Any such new cell would suffer the same fate. The space can remain empty, in which case the organ shrinks, the capillaries draw closer together, new cells are now deprived of the VEGF formally produced by the now missing cell, so capillaries ablate and the hypoxic zone reforms. This could result in a general shrinkage of the affected tissues. In tissues that support fibrosis, relatively inert collagen fibers can fill the space. Since the metabolic requirements of the body for the particular organ in question are not reduced, the organ may attempt to grow larger, but now with a significant fibrous content. This may result in fibrotic hypertrophy, such as of the heart and liver. Some organs, such as the brain, cannot grow larger or smaller because the 3 dimensional connectivity of nerves and blood vessels are important, and cannot be continuously and simultaneously mapped onto an asymmetrically shrinking brain. The space must be filled with something, and β -amyloid might be the (not so inert) space filler. The kidney cannot grow larger because of the renal capsule, so the number of living cells becomes smaller and they are replaced with fibrotic tissue. If the dead cells are cleared, the tissue shrinks, and the ratio of NO/O₂ goes down again, and the capillaries again become sparser. This may set up the vicious circle of end stage renal disease, congestive heart failure/cardiac hypertrophy, primary biliary cirrhosis, Alzheimer's disease, atherosclerosis, inflammatory bowel disease, hypertrophic scar formation, and the multiple connective tissue diseases starting with Raynaud's phenomena and ending with Systemic Sclerosis and primary Sjogren's syndrome where capillary rarefaction is also observed. Reduction in basal NO levels through chronic inhibition of NOS with L-NAME leads to generalized fibrosis of the heart and kidneys⁶⁸ so that low basal NO leads to fibrotic hypertrophy.

Capillary density as factor in appetite regulation

In another embodiment of the invention, it is appreciated that capillary rarefaction affects a subjects ability to control their appetite. Capillary rarefaction is observed in 5 the brains of aged humans and animals.⁶⁹ Capillary rarefaction is associated with declines in circulating growth factors including insulin like growth factor-1.⁷⁰ Neurogenesis in the adult brain is coordinated with angiogenesis.⁷¹ Since the brain regulates many homeostatic functions, increased diffusion lengths between capillaries to control elements of the brain might be “interpreted” as inadequate blood 10 concentrations of those species. The flux of glucose in the brain is quite close to normal metabolic needs, where glucose flux is only 50 to 75% greater than glucose consumption and the glucose transporters across the blood brain barrier are saturable, steriospecific and independent of energy or ion gradients.⁷² A large part of the regulation of appetite is mediated through the brain, and capillary rarefaction may 15 cause an adequate blood concentration of “nutrients” (or marker compounds proportional to “nutrients”) to be interpreted as insufficient. This may be one cause of the epidemic of obesity. Individuals who cannot control their appetite might simply have too long a path between their capillaries and the brain cells that trigger appetite. Their brains might be telling them they are “starving”, because those brain cells that 20 are a little bit too far from a capillary are “starving”. This may not result simply from the longer diffusion path, but by consumption of the “nutrient” by the intervening cells. When cells are hypoxic, and unable to derive ATP from oxidative glycolysis, they instead generate ATP through anaerobic glycolysis. The amounts of glucose required to support metabolism through anaerobic glycolysis is 19 times greater than 25 through oxidative glycolysis.⁷³ Thus a single hypoxic cell could consume as much glucose as 19 non-hypoxic cells. If even a few partially hypoxic cells were between a “glucose sensing cell” and the capillary which is the glucose source, the “glucose sensing cell” would necessarily receive an erroneously low reading. While neurons generate ATP only through oxidative phosphorylation, other brain cells such as 30 astrocytes can also generate ATP through anaerobic glycolysis.⁷⁴ A few hypoxic astrocytes in proximity to a neuron would likely deprive that neuron of glucose. The craving for sugar and carbohydrate that plague many people may derive from specific neurons being deprived of glucose due to nearby hypoxic astrocytes. The elevated

blood sugar may be an attempt to get more glucose to those cells, but because the glucose transporters are saturable and the pathway is blocked by too many hypoxic astrocytes, it may not be possible for blood sugar to be high enough. The association of obesity with chronic degenerative diseases may not be because obesity "causes" them, but because the thing that does cause obesity (capillary rarefaction) also causes degenerative diseases. Exercise does increase basal NO levels in normal healthy and hypercholesterolemic individuals,⁷⁵ so the positive effects of exercise on obesity could be mediated through nitric oxide mediated angiogenesis. Induction of ketosis, either through starvation or through a ketogenic diet (low carbohydrate) causes the liver to generate ketone bodies acetoacetate and β-hydroxybutyrate from lipids. These ketone bodies circulate and are used by neurons instead of glucose in oxidative phosphorylation. A ketogenic diet increases the threshold for seizure induction through electroshock, hyperbaric oxygen, and chemically induced seizures. A ketogenic diet has been used to treat epilepsy for over half a century.⁷⁶ It has been suggested that the anti-seizure effects of a ketogenic diet are due to greater neuron energy reserves.⁷⁷ The appetite suppression effects of a ketogenic diet may similarly derive from greater neuron energy reserves.

During the course of using the invention, the inventor has noticed a pronounced reduction in appetite, and has lost ~30 pounds over the course of a year, simply by eating less without pronounced discomfort. While the inventor was formally unable to function while skipping meals, he is now able to skip multiple meals with no loss in ability to function either mentally or physically.

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Capillary rarefaction as a cause of non-insulin dependent diabetes

According to another aspect of the invention, it is appreciated that capillary rarefaction may be a cause of non-insulin dependent diabetes. Non-insulin dependent diabetes (NIDDM) is also known as the Metabolic Syndrome or Diabetes type 2, and is characterized by insulin resistance. The sensitivity of the body to insulin is reduced, and insulin levels increase. The "cause" remains unknown in spite of intense research. It is observed in all developed regions of the World, across many

cultures and many ethnic groups. People with NIDDM have high blood glucose, high blood triglycerides, are typically obese, hypertensive, and typically have significant visceral fat.

- 5 Other symptoms accompany NIDDM, which the inventor believes point to capillary rarefaction as the cause. In a study of 40 men, with and without NIDDM, obese (BMI 29) and lean (BMI 24) (10 of each), blood lactate levels at rest were 1.78, 2.26, 2.42, and 2.76 (mM/L) for lean men without, obese men without, lean men with NIDDM, obese men with NIDDM respectively.⁷⁸ Lactate is a measure of anaerobic glycolysis. When O₂ is insufficient to generate ATP through oxidative phosphorylation, cells can produce ATP through anaerobic glycolysis. One of the products of anaerobic glycolysis is lactate, which must be exported from the cells, otherwise the pH drops and function is compromised. Blood lactate is commonly measured in exercise studies, where an increase indicates the work load at which maximum oxidative work can be done. Higher levels of lactate at rest would indicate increased anaerobic glycolysis at rest, which is consistent with capillary rarefaction. It is interesting to note that lean diabetic men had higher lactate than obese non-diabetic men.
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- 15
- 20 Muscle cells of NIDDM individuals have higher ratios of glycolytic to oxidative enzymes than do non-NIDDM individuals. NIDDM individuals thus derive a greater fraction of their muscle energy from anaerobic glycolysis than from oxidative phosphorylation.⁷⁹
- 25 Measurement of muscle pH and phosphate species with MRI before and during muscle activity has demonstrated that men with well controlled diabetes type 1 have altered muscle physiology. Diabetic men have reduced oxidative capacity, and derive a greater fraction of their ATP from anaerobic glycolysis, and this difference is apparent even at rest.⁸⁰ This study is interesting because it measures lactate production in vivo through pH changes. In their study they noted that some individuals had two distinct populations of muscle cells with different pH and hence lactate production, 4 of 10 diabetics and 2 of 10 non-diabetics. In their study they simply averaged the values, however, distinct populations of cells with different
- 30

lactate production is indicative of different oxidative phosphorylation capacity and hence different O₂ supply.

Woman with NIDDM have decreased VO_{2max} when compared with both lean and
5 obese controls. This reduced VO_{2max} is apparent even in the absence of any cardiovascular complications.⁸¹ Women with NIDDM had lower peak work production and greater blood lactate levels, both at rest and during exercise.

These observations of increased anaerobic glycolysis in people with both type 1 and
10 type 2 diabetes are consistent with chronic decreased O₂ delivery to the peripheral tissues. That this increased anaerobic glycolysis is observed at rest, when metabolic demand is at a minimum, indicates that this decreased O₂ delivery is chronic.

Treatment of liver inflammation with AAOB

15 Primary biliary cirrhosis is associated with Raynaud's phenomena, pruritus, sicca syndrome, osteoporosis, portal hypertension, neuropathy, and pancreatic insufficiency,⁸² and liver abnormalities are associated with rheumatic diseases.⁸³ Elevated liver enzymes are a symptom of liver inflammation, and elevated liver enzymes are observed as an early symptom of "asymptomatic" primary biliary cirrhosis.⁸⁴

20 Elevated liver enzymes are commonly seen in patients with collagen diseases, including biliary cirrhosis, autoimmune hepatitis and nodular regenerative hyperplasia of the liver matoid arthritis (RA), polymyositis and dermatomyositis (PM and DM), systemic sclerosis (SSc), mixed connective tissue disease (MCTD) and polyarteritis nodosa (PAN).⁸⁵

25 The progression of primary biliary cirrhosis is characterized by 4 stages, first is the inflammatory destruction of the intrahepatic small bile ducts due to previously unknown causes, followed by the proliferation of ductules and/or piecemeal necrosis, followed by fibrosis and/or bridging necrosis, followed by cirrhosis. There is a correlation between cirrhosis of the liver and liver cancer.⁸⁶ A variety of autoimmune

connective tissue diseases are associated with primary biliary cirrhosis, including Sjögren's syndrome, scleroderma, CREST syndrome (calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, or telangiectasia), inflammatory arthritis, or thyroid disease.⁸⁷

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The treatment of choice for primary biliary cirrhosis is oral ursodeoxycholic acid. This is a hydrophilic bile salt that displaces other more toxic hydrophobic bile salts in the hepatic circulation. While the mechanism is not fully understood, a component of the therapeutic effects may derive from reduced metabolic load on the liver through 10 reduced bile synthesis.

While anti-mitochondrial anti-bodies are usually present in primary biliary cirrhosis, 5-10% of patients with PBC do not have such antibodies moreover, most of these 15 patients have autoimmune antibodies to smooth muscle or nuclear factors. However, immunosuppressant therapy is not as effective at slowing the progression of PBC as oral ursodeoxycholic acid is.⁸⁸ This indicates that autoimmune antibodies are not the cause of PBC, but instead are a consequence of some other cause.

In one embodiment of the invention, application of AAOB to the scalp and body of an 20 individual resulted in a lowering of liver enzymes. Figure 1 shows a plot of liver enzymes, alanine transaminase levels (SGPT or ALT) for a single individual both before and during application of AAOB to the scalp and body. Following application of the AAOB, the SGPT level dropped to the lowest point in nearly 20 years. Nitric oxide is known to trigger the initiation of liver regeneration.⁸⁹ Thus the application of 25 AAOB is shown to be effective in reducing elevated liver enzymes and the chronic liver inflammation that elevated liver enzymes indicate. While there is only sparse data to indicate the time scale of the reduction in liver enzymes following application of AAOB, it appears to not be instantaneous. A gradual reduction is consistent with the gradual resolution of long standing capillary rarefaction through capillary 30 remodeling following increased basal NO levels.

Reducing liver inflammation slows the progression of PBC and of other liver diseases and reduces the progression to cirrhosis which is associated with liver cancer.

- 5 In another aspect of the invention, it is appreciated that "hypoxia" used to regulate capillary density may occur during sleep. Though not being bound by one particular theory, the drop in blood pressure and in blood flow rate that normally occurs during sleep is one of the body's normal "housekeeping" functions, and serves to reset the oxygen diffusion resistance between the capillaries and the cells that those capillaries support. The normal drop in blood pressure at night is attributed to increased NO, where inhibition of NOS with L-NNA abolishes wake-sleep differences in cerebral blood flow.⁹⁰ Inhibition of NOS in rats inhibits normal sleep.⁹¹ Humming greatly increases nasal NO by increase gas exchange with the sinuses where NO is produced.⁹² A number of the disorders associated with capillary rarefaction are also 10 associated with disordered breathing at night, either snoring or sleep apnea. Obesity, age, cardiovascular disease,⁹³ hypertension,⁹⁴ rheumatoid arthritis,⁹⁵ are all associated 15 with disordered breathing during sleep. Therefore, it is appreciated that high levels of NO may be advantageous during sleep, and sweating at night as well as snoring may both physiological mechanisms to increase basal NO. High levels of NO during 20 sleep increase the NO/O₂ ratio and so increase the "hypoxia" signal.

The theory that capillary spacing is determined during sleep is supported by the exercise training philosophy of "living high-training low," where athletes train at low altitude, but go to high altitude to live and sleep⁹⁶. Training at low altitude allows 25 greater metabolic load on the muscles being trained, where hypoxia is induced by near maximal metabolic load. Inducing hypoxia by reducing O₂ supply at night might not be effective for muscle because of their high capacity for anaerobic respiration and high levels of O₂ storing myoglobin. However, avoiding subjecting muscle to nightly hypoxia with insufficient NO might be an explanation for why cancers of muscle are 30 so rare. Hypoxia in organs not under conscious control cannot be induced voluntarily through exercise. For example, erythropoietin is produced by the kidney under conditions of "hypoxia" and regulates the production of erythrocytes and Hct. Erythropoietin is up regulated almost immediately with hypobaric hypoxia with

nearly a 50% increase after 6 hours at 2800 meters.⁹⁷ EPO is commonly given to kidney dialysis patients to compensate for the loss of EPO from diseased or missing kidneys and to raise hematocrit. However, raising hematocrit close to the "normal" range increases mortality over lower levels. In a randomized study of 1233 patients, 5 raising Hct to 42% resulted in a 22% greater death rate over 29 months than patients with Hct raised to 30% (183 vs. 150 deaths). The causes of death were similar in the two groups, and characteristic of dialysis patients, there were simply more deaths in the high Hct group.⁹⁸ I suggest that the elevated Hct decreased the basal NO level, and the increased death rate was due to decreased basal NO. The causes of death 10 were similar because both groups actually have low NO levels, it is low NO that brought about the kidney damage in the first place. While low Hct is "bad", low NO is bad too. Without a good way to increase basal NO levels (until now), balancing the increased O₂ capacity of the blood with the decreased NO concentration is a difficult treatment choice.

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Alzheimer's Disease

There is substantial evidence that Alzheimer's disease (AD) is a microvascular disorder with neurological degeneration secondary to hypoperfusion, resulting in part 20 from insufficient nitric oxide.⁹⁹ AD does not occur in all individuals, and it does not occur in single or even a few episodes of hypoperfusion, rather it occurs over time, sometimes over many years. The course of Alzheimer's, while inexorable and monotonic, is not steady, and is not associated with known episodes of hypoperfusion or syncope. In the early stages there can be considerable variability in degree of 25 neuropathy and in rate of decline. That is one factor that can make the diagnosis of Alzheimer's difficult in the early stages.

Levels of ischemia sufficient to produce the levels of oxidative damage observed in AD due to hypoperfusion would produce noticeable contemporaneous mental effects. 30 Levels of hypoxia and ischemia not producing oxidative damage are noticeable. Levels of hypoperfusion resulting in confusion or syncope are typically not reported by Alzheimer's patients, so the oxidative damage must have occurred during a non-reportable time, it may have occurred during sleep.

During sleep, the metabolism of all parts of the body is reduced. The blood pressure falls and the blood flow decreases. The velocity of blood flow throughout the body decreases, and with less shear at the vessel walls eNOS is down regulated and NO production by eNOS is reduced. The energy demands of the brain are reduced. The brain however is still quite active and still requires substantial blood flow.

Hypothermia is known to reduce cerebral damage during ischemic events. Hypothermia both during and even after such events reduces brain damage by reducing the reperfusion injury. Sleep normally causes a drop in body temperature of 0.5-0.7 °C¹⁰⁰. Mild hypothermia during sleep would independently reduce energy needs of the brain and would reduce the ischemic threshold for damage. The basal metabolism rises approximately 14% for every 1°C of fever, so the “normal” reduction, during sleep, of 0.5-0.7 °C is a reduction of 7 to 10% in metabolic rate.

NO is known to be necessary in the reduction of basal temperature due to hypoxia. When NO synthesis is inhibited with N-nitro-L arginine (L-NNA) the reduction in basal temperature following hypoxia is greatly diminished.¹⁰¹

The reports of a “protective effect” on Alzheimer’s associated with non-steroidal anti-inflammatory drugs (NSAIDs), could, in part, result from their effect in lowering body temperature.

The epidemiology of Alzheimer’s is well studied in developed countries but much less so in underdeveloped countries. Reliable and consistent differential diagnosis across many patients, many physicians, and many cultures is difficult and perhaps fraught with error. That said, according to the present theory that the causal events of hypoxia occur during sleep, then the incidence should increase with increasing sleeping temperatures. Tables 1 and 2 show the incidence of Alzheimer’s reported in a review article by Suh and Shah¹⁰². The temperatures were taken from tabulated monthly averages¹⁰³. The data was divided into two sets, a “developed” and an “undeveloped” group. Beijing was included in both, with 1987 data as “undeveloped” and 1999 data as “developed”. The two groups were divided on the basis of perceived

per capita water consumption for bathing. The relevant population is the populations at risk for AD, the elderly. That population is likely to lag behind others in the adoption of new bathing practices.

- 5 Table 1 shows maximum and minimum average monthly temperatures and incidence of Alzheimer's Disease and Total Dementia for undeveloped cities. Table 2 shows maximum and minimum average monthly temperatures and incidence of Alzheimer's Disease and Total Dementia for developed cities.

10

Table 1

Undeveloped	Date of	Hottest	Average	Average	Prevalence	Prevalence
City	Study	month	High	Low	Alzheimer's	Total
			Temperature	Temperature	Disease	Dementia
Beijing	1987	July	87.4	70.9	0.4	0.8
Shanghai	1990	July	88.9	76.6	3	4.6
Hong Kong	1998	July	92.7	74.5	4	6.1
Taiwan (Taipei)	1998	July	90	77.9	2.3	4
Ibadan (Lagos)	1997	February	91.8	75.4	1.1	1.4
Kerala (Bangalore)	1998	April	93.6	71.2	1.4	3.4
Tokyo	1982	August	87.6	75.2	1.2	4.8

Okinawa	1995	July	88	79	3.1	6.7
Hiroshima	1999	August	87.6	74.5	2.9	7.2
Aichi (Nagoya)	1986	August	90	74.3	2.4	5.8
Wuhan (Wuhu)	1981	July	88.9	76.6	0.1	0.5

Table 2

5

Developed City	Date of study	Hottest month	Average High	Average Low	Prevalence Alzheimer's Disease	Prevalence Total Dementia
Beijing	1999	July	87.4	70.9	4.8	7.8
Boston	1989	July	81.8	65.1	8.7	10.3
Odense	1997	August	69.4	52.2	4.7	7.1
London	1990	July	71.1	52.3	3.1	4.7
Stockholm	1991	July	71.4	56.1	6	11.9
Rotterdam (Amsterdam)	1995	July	85.5	43.7	4.5	6.3

- The bathing practice believed to be important is the washing of the head and scalp with detergents which washes off the natural population of autotrophic ammonia oxidizing bacteria which produce nitric oxide for absorption into the scalp. In one aspect of the invention, not washing one's head is protective regarding AD, the populations likely show mixed behavior with different patterns of head washing. In developed cities with abundant shampoo products and clean hot water, washing one's head is common, and the population that washes their head less frequently than once per week is likely small. Washing one's head is common in the developed cities, and the population that washes their head less than once per week is likely small. In the undeveloped cities, there are likely still a considerable number that wash their head frequently enough to be essentially free from autotrophic bacteria. That part of the population may represent the majority of the AD cases in the undeveloped cities.
- 15 The data is plotted in Figure 2, which shows the incidence of AD versus minimum temperature during the hottest month (i.e. temperature at night during sleep). The two data sets seem to fall into two groups, with increased minimum temperature correlating with increased incidence of AD, but with a different slope and intercept. The undeveloped intercept is around 70 F. Any intercept for the "developed" group would be off the chart, and would be unrealistic because heating would be used to raise the temperature into a "comfort zone". While the progression of AD in undeveloped regions may show seasonality due to different sleeping temperatures, in developed regions, the intercept is below the minimum temperature that most people sleep at irrespective of outside temperature.
- 25 According to one aspect of the invention, it is appreciated that a factor in the current high incidence of AD is the improvement in shampoo technology that occurred in the early 1970's allowing one to shampoo often, even daily. Prior to that time, if one were to shampoo everyday, one's hair would "turn to straw", and would be unaesthetic. It was the development of "conditioning" shampoos that allowed daily hair washing. A chart of the number of US patents issued on shampoo is shown in Figure 2. There is a large surge in the early 1970's. According to one aspect of the

current invention, the current epidemic of obesity, diabetes, and AD derives from the development and adoption of conditioning shampoos and their frequent use.

Other adverse health effects that are associated with hypertension may also be
5 consequences of low basal NO. The decreased response to vasodilatation is also consistent with low basal NO. NO is a diffusible molecule that diffuses from a source to a sensor site where it has the signaling effect. With low NO levels, every NO source must produce more NO to generate an equivalent NO signal of a certain intensity a certain distance away. NO diffuses in 3 dimensions and the whole volume
10 within that diffusion range must be raised to the level that will give the proper signal at the sensor location. This may result in higher NO levels at the source and between the source and the sensor. Adverse local effects of elevated NO near a source may then arise from too low a NO background. There is some evidence that this scenario actual occurs. In rat pancreatic islets, inhibition of NOS with L-NAME increases
15 total NO production through the induction of iNOS.¹⁰⁴ Increasing NO by increasing NOS activity will only work up to some limit. When NOS is activated but is not supplied with sufficient tetrahydrobiopterin (BH4) or L-arginine, it becomes “uncoupled” and generates superoxide (O₂⁻) instead of NO¹⁰⁵. This O₂⁻ may then destroy NO. Attempting to produce NO at a rate that exceeds the supply of BH4 or L-
20 arginine may instead decrease NO levels. This may result in positive feedback where low NO levels are made worse by stimulation of NOS, and uncoupled NOS generates significant O₂⁻ which causes local reactive oxygen species (ROS) damage such as is observed in atherosclerosis, end stage renal disease, Alzheimer’s, and diabetes.

25 Aging

Agents to slow the progression of aging have been searched for since antiquity, but to little effect. The only demonstrated treatment that prolongs life is calorie restriction, where restricting food intake to 70% of ad lib controls, prolongs life in sedentary rats
30 from 858 to 1,051 days, almost 25%.¹⁰⁶ The link between calorie restriction and prolonged life is well established, however, the causal mechanism is not. Examination of liver mitochondrial enzymes in rats indicates a reduction in H₂O₂ production due to reduced complex I activity associated with calorie restriction.¹⁰⁷

H₂O₂ is produced by dismutation of O₂⁻, which is a major ROS produced by the mitochondria during respiration. The main source of O₂⁻ has been suggested to be complex I which catalyzes the NAD/NADH redox couple¹⁰⁸ by reverse flow of electrons from complex III, the site of succinate reduction.¹⁰⁹ The free radical theory of aging postulates, that free radical damage to cellular DNA, antioxidant systems and DNA repair systems accumulates with age and when critical systems are damaged beyond repair, death ensues.¹¹⁰

In addition to free radical damage leading to senescence, there is also programmed senescence based on the length of telomeres which shorten with each cell division. NO has been demonstrated to activate telomerase and to delay senescence of endothelial cells.¹¹¹ Low basal NO will increase basal metabolic rate by disinhibition of cytochrome oxidase. Increased basal metabolism will also increase cell turn-over and growth rate. Capillary rarefaction, by inducing chronic hypoxia may increase free radical damage and may also increase cell turn-over, and so accelerate aging by both mechanisms.

In another aspect of the invention, it is appreciated that AAOB affects the age of puberty onset. An interesting observation in human aging is that the age of menarche declines as a region becomes more developed. A number of factors have been used to explain this, however the correlation that “best” fits the data, is an inverse relationship with illiteracy rate.¹¹² However, in the US, the median ages of menarche in 1974 were 12.9 and 12.7 years for black and white girls respectively.¹¹³ In 1994 they were 12.1 and 12.5 years. It has been suggested that this decline in age of menarche relates to dietary practices, in particular to increased fat in the diet. However, from 1965 to 1995, the percentage of fat in the diet of 11-18 year olds actually dropped from 38.7% to 32.7%.¹¹⁴ In Norway, the age of menarche has dropped from 16.9 years in 1850 to 13.3 years in 1950. The change is quite linear over time. In the US, from 1910 to 1950, the drop was from 14 to 13,¹¹⁵ also quite linear, with no increase observed during the Depression, when presumably food availability would have been less. The age of puberty may be actually due to the loss of AAOB through bathing, and not due to increased availability of food. The association of early menarche with literacy rate may be due to the adoption of the Western notion that “cleanliness is next to

godliness." Disease is not associated with dirt, disease is associated with pathogens, which may or may not be associated with dirt. The elimination of diarrheal diseases due to modern sanitation may not be due to increased bathing, but may be due to sanitary disposal of pathogen containing fecal matter, and the prevention of the 5 contamination of the water supply by pathogen containing wastes.

Life expectancy generally increases with economic development. This increase is due to a number of factors. Infant mortality decreases due to declining starvation, diarrheal diseases, and other infections. Life expectancy of adults increases due to 10 better access to health care. However, some developed countries have started to see the life expectancy of their aged populations actually decline. In the Netherlands, the life expectancy at age 85 has declined in men since the 1980's and in both sexes since 1985/89.¹¹⁶ While there are increases due to mental disorders (presumably Alzheimer's Disease), cancer and diabetes, and chronic obstruction pulmonary 15 disease, all conditions expected to be exacerbated by a reduction in basal NO levels.

Allergies and autoimmune disorders

In another aspect of this invention, it is appreciated that autotrophic ammonia 20 oxidizing bacteria may produce protective aspects for allergies and autoimmune disorders. The incidence of allergy among children has been increasing throughout the developed world and asthma is now the most common chronic disease of childhood. No clear explanation of the different incidence of allergies and asthma among different population groups has been proposed. The data is quite complex and 25 seemingly contradictory. Autoimmune disorders are also common. The best known is perhaps Diabetes Type 1, which results from the destruction of the insulin producing cells in the pancreas by the immune system. Recurrent pregnancy loss is also associated with autoimmune disorders where the number of positive autoimmune antibodies correlated positively with numbers recurrent pregnancy losses.¹¹⁷ Systemic 30 Sclerosis, Primary Biliary Cirrhosis, autoimmune hepatitis, and the various rheumatic disorders are other examples of autoimmune disorders.

- In general, the incidence of allergies increases with affluence, both as the affluence of a population increases through development, and within a population the incidence is higher in the most affluent group. However, in the US, the incidence of asthma in urban African Americans is three times that of suburban children.¹¹⁸ In contrast,
- 5 Swedish conscripts born in Africa show lower allergy symptoms than those of African decent born in Sweden¹¹⁹. This last paper is interesting because it shows significant differences in allergy incidence based on "socio-economic status" (as measured by >12 years maternal education) for those of "tropical decent", (those with maternal birth in Africa, Latin America or Asia) for both those born in Sweden and those born outside of Sweden. Interestingly, there is much less difference based on "socio-
- 10 economic status" for those with maternal birth in "temperate" regions (Eastern, Western Europe, and Sweden). Those with mothers from intermediate regions (Middle East, Southern Europe) exhibit higher allergy with "socio-economic status," but only for those born in Sweden. The incidence of asthma in those of African
- 15 decent of "high" "socio-economic status" born in Sweden is 2.9 times greater than Swedes, roughly the same ratio seen in the US between urban African Americans and suburban (presumably Caucasian) children. Low "socio-economic status" reduces the incidence to only 1.1 times that of low "socio-economic" Swedes. Being born outside of Sweden has little protective value for high "socio-economic status" the incidence
- 20 still being 2.5 times greater. However, being of low "socio-economic status" and being born outside of Sweden confers substantial protection, the incidence being only 0.56 that of Swedes. Thus there is a 5 fold difference in incidence of asthma for those of African decent depending on place of birth.
- 25 In rural Bavaria Germany, it was found that there was a correlation between the type of fuel used for domestic heating and the development of asthma and other allergies.¹²⁰ Heating with coal or wood (compared with central heating) was found to be protective. It was suggested that perhaps cooler bedroom temperatures might explain less sensitization to dust mites, however there was also less sensitization to
- 30 cats, dogs and pollen. The percentage of homes with cats and with dogs was greater in the coal/wood group. The "socio-economic status" was lower in the coal/wood group.

Observations such as these have led people to propose the “Hygiene Hypothesis” where increased exposure to allergens or diseases during childhood is believed responsible for protective effects regarding the development of later allergies.

However, a consensus statement by a number of professionals at a conference devoted
5 to the Hygiene Hypothesis stated that the data remain conflicting, and there is no indication of which microbe or other agent might be responsible for the protective effects.¹²¹

Application of AAOB has been found to actually reverse a long standing allergy,
10 namely seasonal hay fever (n of 1). The presence/absence of AAOB may explain the “contradictory” data in the literature and demonstrate that it is not contradictory at all. Virtually all studies may be explained through the causal mechanism described here, as is the reason for the sharply increased incidence of allergies for those of tropical decent when born and living in the developed world. It may also explain why low
15 economic status is especially protective when living in regions where bathing practices are a function of economic status. The rural Germans who heated with coal/wood, likely didn’t have copious running hot water with which to bathe. It was not how they heated their home that was protective, but instead the shortage of hot water with which to bathe.

20

The reason that this class of agent has been so elusive is that it does not cause any disease. In fact, the agent cannot cause disease (probably not even in immunocompromised individuals) because it is autotrophic ammonia oxidizing bacteria (AAOB). AAOB are obligate autotrophic bacteria^{122, 123}. They derive all
25 metabolic energy only from the oxidation of ammonia to nitrite with nitric oxide (NO) as an intermediate product in their respiration chain. They derive virtually all carbon by fixing carbon dioxide and are incapable of utilizing carbon sources other than a few simple molecules. The complete genome of one of them (*Nitrosomonas europaea*) has been sequenced and it has only 2460 genes that code for proteins.¹²⁴
30 From an inspection of the genome, it is clear that these bacteria cannot cause disease. There are no genes for toxins or lytic enzymes. They do not have the metabolic machinery to utilize the complex organic compounds such as are found in animal tissues. They do not grow on any heterotrophic media such as is used for isolating

pathogens (all of which are heterotrophic¹²⁵). The only reason they have not been found on the human body is that no one has looked for them with the proper culture media and techniques. They are also slow growing with optimum doubling times of 10 hours compared to 20 minutes for heterotrophs. Attempted isolation on media suitable for heterotrophs would result in overgrowth by heterotrophs because of the 5 30-fold faster doubling rate. They are universally present in all soils where they are responsible for the first step in the oxidation of ammonia into nitrate in the process of nitrification. As autotrophic bacteria, they are incapable of growing anywhere that lacks the substrates they require, ammonia or urea, oxygen, mineral salts. These 10 substrates are abundantly available on the unwashed skin from sweat residues, and in the "wild" and in the absence of frequent bathing with soap, humans would be unable to prevent the colonization of their external skin with these bacteria. Actually, these bacteria are beneficial, and according to an aspect of the invention, it is appreciated that they are commensal, and that many aspects of human physiology have evolved to 15 facilitate the growth of these bacteria and the utilization of the NO they so abundantly produce.

Another factor that perhaps has prevented their isolation is the bathing practices in developed regions. It has become customary to bathe with sufficient frequency so as to 20 prevent the development of body odor. Body odor generally occurs after a few days of not bathing, and the odor compounds are generated by heterotrophic bacteria on the external skin which metabolize exfoliated skin and sweat residues into odiferous compounds. In 3 days, autotrophic bacteria could double approximately 7 times for approximately a 100-fold increase over the post bathing population. In contrast, 25 heterotrophic bacteria could double approximately 200 times for a 10e+60-fold increase. Obviously heterotrophic bacterial growth would be nutrient limited. Assuming similar kinetics of removal through bathing of autotrophic and heterotrophic bacteria, controlling heterotrophic bacteria though bathing would reduce autotrophic bacteria to low, perhaps undetectable levels.

30

In one embodiment of the invention, it is appreciated that a sufficient population of AAOB on the skin substantially suppresses body odor due to heterotrophic bacteria. The inventor has applied AAOB to his skin and has refrained from bathing for 15

months now, including two summers. There is little body odor associated with sweating. In fact, sweating may decrease body odor by nourishing the AAOB and enhancing their production of NO and nitrite which suppress heterotrophic bacteria.. During the winter, with decreased sweating due to low ambient temperatures, there
5 was an increase in odor. However, with increased clothing, (wearing sweaters) the inventor was able to increase basal sweating and reduce body odor to near zero again. There has been no incidents of itching, rashes, skin infections, or athlete's foot infection, and substantially no foot odor.

- 10 The AAOB produce nitric oxide as an intermediate in their normal metabolism,¹²⁶ and one strain had optimum growth at concentrations of NO in air around 100 ppm¹²⁷ (highest level tested in this study). They can tolerate higher levels. With other strains, there was no decline in NH₃ consumption from 0 to 600 ppm (anaerobic in Ar plus CO₂) but it declined by 1/3 at 1000 ppm NO¹²⁸. Most are aerobic, but some
15 strains can utilize nitrite or nitrate in addition to oxygen which increases the NO production. 1000 ppm NO in air corresponds to about 2 μM/L in aqueous solution. The strain used by the inventor has produced a measured NO concentration of 2.2 μM/L. Most studies of AAOB metabolism have been motivated by their utilization in waste water treatment processes for ammonia and nitrate removal from waste water.
20 Operation of waste water treatment facilities at hundreds of ppm NO is undesirable, so it is not unexpected that the physiology of these bacteria under those conditions has not been well studied.

One mechanism by which AAOB may exert their protective effect on allergies and
25 autoimmune disorders is through the production of nitric oxide, primarily through the regulatory inhibition of NF-?B and the prevention of activation of immune cells and the induction of inflammatory reactions. NF-?B is a transcription factor that up regulates gene expression and many of these genes are associated with inflammation and the immune response including genes which cause the release of cytokines,
30 chemokines, and various adhesion factors. These various immune factors cause the migration of immune cells to the site of their release resulting in the inflammation response.¹²⁹ Constitutive NO production has been shown to tonically inhibit NF-?B by stabilizing I?Ba (an inhibitor of NF-?B) by preventing I?Ba degradation.¹³⁰

Allergy, asthma, and autoimmune disorders are characterized by an inappropriate, hyper response of the immune system to a particular antigen. This is thought to derive first from an initial "priming" of T-cells either in utero or shortly after birth, 5 followed by priming to a TH2 phenotype, followed by a skewing and polarization of the TH1/TH2 to a TH2 (allergenic) type.¹³¹

Administration of an NO donor has been shown to prevent the development of experimental allergic encephalomyelitis in rats.¹³² In this study, it was demonstrated 10 that administering an NO donor, reduced the infiltration of macrophages into the central nervous system, reduced the proliferation of blood mononuclear cells, and increased apoptosis of blood mononuclear cells. All of these results are expected to reduce the extent and severity of the induced autoimmune response.

15 Allergen exposure is a necessary aspect of sensitization, however there is little evidence that incidence of allergy is directly related to allergen exposure. Exposure to similar quantities of allergens does not always produce similar levels of allergy. Similar levels of asthma occur in populations with very different exposures to the same and different allergens.¹³³ In a comparison of East and West German levels of 20 allergens prior to unification and subsequent atopic sensitization, the highest exposure levels were in East Germany and the highest levels of atopic sensitization were in West Germany.¹³⁴ There is good evidence that allergen reduction prevents allergic response in sensitized individuals, but there is not good evidence causally linking magnitude of allergen exposure to sensitization.¹³⁵ For some allergens, there does 25 seem to be a positive dose-response effect (dust mites), but for others, there is an inverse dose-response effect (cat allergies).

According to another aspect of the invention, it is appreciated that inhibition of 30 allergies and autoimmune sensitization may be achieved through topical application of AAOB which produce active NO species in the skin. The exact details of how the immune system works are not fully understood. In general, bacteria, dead or dying cells, foreign organisms, or other debris are first phagocytosed by antigen presenting cells. A major class of these antigen presenting cells are the dendritic cells (DC).¹³⁶

These phagocytosed components are digested into smaller fragments, and these fragments are presented to the surface of the antigen presenting cell along with proteins of the major histocompatibility complexes I and II (MHC I and MHC II).

Immature DC digest the foreign body through either the proteosomal or the

- 5 endosomal pathway. In the proteosomal pathway, proteins (primarily) from the DC cytoplasm are digested and the resulting antigens are bound to the MHC I. In the endosomal pathway foreign bodies are digested and the resulting antigens bound to the MHC II. The antigens bound to the MHC are then transported to the cell surface where they can interact with T helper cells which come in contact with the antigen
10 presenting cell. In general "self-type" antigens are processed through the proteosomal pathway and "foreign-type" antigens through the endosomal pathway, but there is some cross-priming where and become activated by binding simultaneously to the antigen and the major histocompatibility complex. These activated T helper cells, then cause the activation of other immune cells. Nitric oxide inhibits mast cell
15 induced inflammation¹³⁷ and inhibits mast cell adhesion through S-nitrosylation of cysteine residues.¹³⁸ S-nitrosoglutathione (GSNO) strongly down regulated mast cell adhesion. GSNO is the species which would be expected to be formed in the skin from AAOB.

20 Estimate of NO absorption on skin from AAOB

- The motivation for this analysis is to estimate the bioavailability of NO produced by AAOB and absorbed through the skin. The main difference between the lung and the skin as exchange surfaces for gases has to do with the proximity of hemoglobin. In
25 the lung, efficient O₂ loading is required and arterial blood leaving the lung is typically >90% saturated with O₂. Oxygenated Hb destroys NO very rapidly. Deoxygenated Hb also binds NO rapidly, rendering it unavailable. In contrast to the reactions with Hb, the reactions with albumin preserve the vasodilatory activity of NO through the formation of a variety of NO containing species, including S-NO-albumin, as NO physically adsorbed in hydrophobic regions of the albumin
30 molecule¹³⁹, and also as a nitrosating species¹⁴⁰, believed to be N₂O₃¹⁴¹ also adsorbed in hydrophobic regions. This last reference demonstrates that albumin can promptly react with authentic NO and oxygen to form complexes that are stable for minutes and

which slowly release authentic NO, and that these NO-O₂-albumin complexes cause vasodilatation in vivo on rats vasoconstricted with L-NAME. These complexes also cause the nitrosation of diverse materials including low molecular weight thiols. In vitro, blocking the sulphydryl groups prevented formation of S-NO-albumin, but did not prevent the formation of this NO-O₂-albumin nitrosating complex. S-NO-albumin also transnitrosates glutathione, especially in the presence of Cu containing proteins such as ceruloplasmin.¹⁴² S-NO-thiols also release NO, and it is not clear exactly which species, NO, GSNO, other low molecular weight S-NO-thiols or S-NO-albumin are important active species, but perhaps all of them are.

10

- According to one aspect of the invention, it is appreciated that the transport mechanism for moving NO species from the skin to guanylyl cyclase (GC) where it can act is via S-NO-thiols, either S-NO-albumin, GSNO, or other low molecular weight species. The advantages of using the skin as the exchange surface for nitrosylation of albumin are several. First, it would allow the NO to be absorbed into the extravascular plasma substantially without encountering Hb. The lifetime of NO species in plasma without Hb is very long. Second, the external skin is much more tolerant of NOx than is the lung. The outer surface is actually dead, and is continually renewed. If the NO-albumin complexes formed in vitro are the species which transport NO systemically in vivo, then the therapeutic effectiveness of transdermal NO would be many-fold higher than that through inhalation. Third, since the expected active species is an S-NO-thiol, the non-enzymatic oxidation of NO with O₂ does not destroy NO, it converts it to N₂O₃ which is a good nitrosating agent.
- 25 Autotrophic ammonia oxidizing bacteria may be commensal, and humans may have evolved to utilize the NO that they produce, so there should not be any deleterious side effects from their use to raise basal NO levels. According to one aspect of the invention, it is appreciated that many of the diseases of the modern world result from an NO deficiency due to the loss of these bacteria through modern bathing practices.
- 30 positive side effects, particularly in those of recent African decent whose recent ancestors didn't evolve compensatory NO pathways to deal with the loss of NO from AAOB during winter may result from use of AAOB. This may be one reason why the African American community is hit harder by obesity, diabetes, hypertension, asthma,

atherosclerosis, heart disease, end stage renal disease, precocious puberty, etc.

Photochemical dissociation of NO from SNO-thiols is well known,¹⁴³ and the loss of skin and hair pigmentation at high latitudes may derive from a need for increased photochemical dissociation of SNO-thiols in the external skin and not from vitamin D

- 5 metabolism. Sweating on the scalp increases at night, when photo dissociation of SNO-thiols would be at a minimum. Hair becomes white with age, perhaps to allow greater light penetration for photochemical NO release. Tyrosinase, the enzyme that forms melanin is a type-3 copper containing oxidase, a number of which catalyze the formation of SNO-thiols.

10

- The external skin derives all of its metabolic O₂ needs from the external air.¹⁴⁴ There is thus no need for erythrocytes to circulate through those regions, and for the most part, they do not. For the most part the color of skin is due to pigment and erythrocytes. Non pigmented skin is relatively transparent, and the color accurately reflects the circulation of erythrocytes in the surface layers. While the living outer layers of skin derive O₂ from the atmosphere, they derive all other nutrients from the blood. Plasma is blood without erythrocytes, and thus can supply everything except O₂. Since the outer layers of skin are essentially erythrocyte free, but are still actively metabolizing, plasma may be circulating through those outer layers of skin which 15 derive O₂ from the atmosphere. It is in this erythrocyte free skin that conversion of 20 NO to S-NO-albumin occurs.

- The lifetime of NO in the blood is extremely short. NO is rapidly oxidized by O₂Hb, rapidly binds to Hb, is complexed by albumin, is oxidized to N₂O₃ and NO₂ through 25 non-enzymatic reaction with O₂, and also forms S-NO-thiols. A significant site of action of NO is guanylyl cyclase (GC) where the apparent EC₅₀ is about 45 nM/L for rapid (~100 ms) and 20 nM /L for slow (~1 to 10 sec) activation¹⁴⁵. There are significant difficulties in estimating the fraction of an administered dose of an NO source that will reach the target tissues in pharmacological amounts. For example, 30 when inhaled NO is administered at 80 ppm in >90% O₂ (16 μM/min = 14 μM/kg/hr) there is no change in mean arterial pressure. In contrast, sodium nitroprusside (SNP) at 0.9 μM/min (0.75 μM/kg/hr) causes a 25% reduction in mean arterial pressure,¹⁴⁶ indicating that when administering NO through inhalation, the concentration of NO at

the resistance determining vessels does not increase to 20 nM/L and activate GC. Thus SNP is many times more "effective" at delivering "NO active species" to peripheral GC than is inhaled NO.

5 SNP has also been compared to intravenous NO, where intravenous NO, SNP, and S-
NO-glutathione (GSNO) were shown to have relative "maximally effective doses"
administered as bolus infusions in local brachial artery vasodilatation of 6 μ M, 34nM,
and 5 nM respectively.¹⁴⁷ This puts the relative effectiveness of intravenous NO,
SNP, and GSNO at 1:176:1200. There were significant differences in the temporal
10 course of vasodilatation induced through the above treatments. Both the NO and the
GSNO treatments had a more sustained effect than SNP. Thus GSNO is roughly 7
times more "effective" at getting "NO active species" to peripheral GC than is SNP.
Presumably then, a dose of about 0.1 μ M/kg/hr of GSNO would have a vasodilatation
effect equivalent to 0.75 μ M/kg/hr SNP. The basal nitrate excretion is about 1
15 μ M/kg/hr. If we assume that the vasodilatory effects of 0.75 μ M/kg/hr SNP are on the
"same order" as the indigenous NO already produced, then the 0.1 μ M/kg/hr GSNO
represents an increase in "effective NO" of 50 % over basal levels.

20 Copper, either as Cu²⁺ or as ceruloplasmin (CP) (the main Cu containing serum
protein which is present at 0.38 g/L in adult sera and which is 0.32% Cu and contains
94% of the serum copper¹⁴⁸) catalyzes the formation of S-NO-thiols from NO and
thiol containing groups (RSH). CP in sub μ M/L concentrations had activity greater
than that of free Cu²⁺, and in the presence of physiologic chloride concentrations the
activity was approximately doubled¹⁴⁹. A number of other Cu containing enzymes
25 also catalyze the formation of S-NO-R:

30

Table 3

% Conversion RS-NO/NO

	GS-		NAC-	
	NO	+/- S.E.	NO	+/- S.E.
Control	0.27	0.03	0.43	0.02
CuSO ₄	5.61	0.55	16.14	0.24
Human CP	28.9	2.38	50.03	1.83
Laccase	10.42	0.5	6.97	0.83
Ascorbate oxidase	6.03	1.14	3.19	0.15
Azurin	2.15	0.24	1.53	0.23
Cu,Zn-SOD	1.97	0.01	1.91	0.16
Hemocyanin	5.7	0.23	2.75	0.03
Nitrite reductase	1.06	0.05	2.85	0.16

“[Table 3¹⁵⁰ shows] Nitrosothiol-producing activities of various copper-containing proteins. RS-NO was formed in the reaction of reduced glutathione (GSH) (20 μM) or N-acetyl-L-cysteine (NAC) (20 μM) and P-NONOate (10 μM) with or without CuSO₄ or various copper containing proteins. CuSO₄ or copper-containing proteins (protein subunits) were used at a concentration of 2.0 μM. The amount of RS-NO (GS-NO and NAC-NO) reached a plateau or declined when the concentration of CuSO₄ or each copper-containing protein exceeded 2 μM. Data are the means 6 S.E. of four experiments”.

The formation of GSNO from NO and GSH is shown to be approximately 100 times greater in the presence of physiologic concentrations of CP. They also report that CP produced significant GSNO even at nanomolar concentrations of NO.

They also show that in cell culture, murine macrophage cells (RAW264) with iNOS induced by interferon-? and lipopolysaccharide, and supplemented with CP (2 μM/L) in-Krebs-Ringer-phosphate, roughly 1/3 of the oxidized NO species produced, (nitrate, nitrate and RSNO) ended up as recovered NAC-NO. This finding is remarkable. It demonstrates that in the absence of hemoglobin, conversion of authentic NO to RSNO can be quite efficient and as high as 33%.

The Cu content of plasma is variable and is increased under conditions of infection. The Cu and Zn content of burn-wound exudates is considerable with patients with 1/3 of their skin burned, losing 20 to 40% of normal body Cu and 5 to 10% of Zn content in 7 days¹⁵¹. I suggest that the Cu in burn exudates is there to catalyze the conversion of NO into S-NO-thiols. As an aside, if the patients skin were colonized by AAOB, wound exudates which contains urea and Fe, Cu, and Zn that AAOB need, would be converted into NO and nitrite, greatly supplementing the local production of NO by iNOS, without consuming resources (such as O₂ and L-arginine) in the metabolically challenged wound. A high production of NO and nitrite by AAOB on the surface of a wound would be expected to inhibit infection, especially by anaerobic bacteria such as the Clostridia which cause tetanus, gas gangrene, and botulism. The xanthine oxidase content of the skin would increase NO levels by reducing any nitrite produced by the AAOB into NO. Inhibiting the Clostridia which cause botulism food poisoning is the primary reason for the use of nitric oxide (as nitrite) to cure and preserve meat.¹⁵² In a textbook on microbial disease, the author of the chapter on Clostridia, Rubin writes: "In some developing countries the umbilical stump of newborn children is packed with mud or dung to soothe the infant."¹⁵³ Rubin suggests that such a procedure prevents tetanus infection by rendering the wound aerobic however, the actual anti-tetanus agent may be nitric oxide produced by the AAOB bacteria in mud when acting on the ammonia and urea found in dung.

The skin contains 9.2 ppm Fe¹⁵⁴, while whole blood contains 500 ppm Fe and plasma contains 1 ppm Fe¹⁵⁵. The major concentration of hemes in the skin is hemoglobin in the capillaries, which is why the color of skin reflects perfusion. Since the heme content of the skin is at most 2% that of the blood, it would be expected that in the skin, NO would have a lifetime at least 50 times that in the blood. Actually it would be more, because some of the iron is present not as hemes, but as iron complexes that are not reactive toward NO. The skin represents 18% of adult body weight and contains 23% of the body's albumin (about 65 g for 70 kg male). NO reacts with O₂Hb to form nitrite and nitrate which are inactive. NO reacts with thiols to form S-NO-thiols, and has a non-enzymatic reaction with O₂ to form NO₂. NO₂ can readily nitrosate thiols too. The non-enzymatic reaction with O₂ thus does not remove and prevent NO from forming S-NO-thiols. A reaction in determining the production of

S-NO-albumin in the skin is the destruction of NO by O₂Hb. All of the NO that is not so destroyed should instead form S-NO-albumin. Actually, NO that is converted into nitrite or nitrate can be “recycled” into NO by xanthine oxidoreductase¹⁵⁶. Similarly, nitrite and nitrate can be “recycled” by the AAOB, which can use nitrite or 5 nitrate instead of oxygen under anaerobic conditions.

The oxygen permeability of the stratum corneum of the skin is about 3.7E-7 ml/m/min/mmHg and 1.3 E-6 in the living portion¹⁵⁷. The stratum corneum is about 10 to 20 microns thick. The viable epidermis and the stratum papillare extend to 10 about 250 microns, and both are supplied with O₂ from the external atmosphere and not from the vasculature¹⁵⁸. The permeability of both tissues increases as the water content increases. The hydration state of the stratum corneum was not specified, so a higher permeability might be expected on a sweating scalp.

15 The physical properties of O₂ and NO are quite similar, including the partitioning between aqueous and lipid phases, so the permeability of skin to NO is similar to that of O₂, however, NO is a lighter molecule which has greater solubility in water and other fluids. If we assume the permeabilities vary as does the solubility in water, then NO would have a 1.5 greater permeability than O₂. If the internal NO 20 concentration exceeded 20 nM/L, then GC would be activated, the local vessels would dilate, blood flow would increase, and the NO in excess of 20 nM/L would be convected away or oxidized by O₂Hb. 20 nM/L corresponds to a gas phase concentration of 10 ppm. Thus we can use 10 ppm as the internal NO concentration. The NO flux through the skin would then be proportional to the concentration 25 difference, the permeability of the skin, and the thickness of the various layers.

The main unknowns are the thickness of skin that the NO must diffuse through to reach the plasma where it is converted into RSNO species. The glutathione (GSH) content of the stratum corneum of hairless mice is about 100 pM/μg protein, or about 30 0.3%.¹⁵⁹ The second unknown is the efficiency of conversion of NO to RSNO.

The diffusion resistance of an external “biofilm” would be easy to adjust therapeutically. Any gel forming material such as KY jelly or various hair gels would

present a diffusion barrier to NO loss through the hair to ambient air. The NO level in the skin cannot greatly exceed 20 nM/L because that level activates GC and would cause local vasodilatation and oxidative destruction of excess NO. The NO concentration at the stratum corneum will increase until it either diffuses away, or the bacteria producing it are inhibited. Which will happen first depends primarily on the external resistance which is easily adjusted.

The scalp can be modeled as a bioreactor generating NO from injected sweat. However, the only loss mechanisms from the scalp biofilm are diffusion through the scalp and diffusion to the ambient air. The biofilm can be thought of as a reactor cycling between dry aerobic and wet anaerobic conditions. NH₃ would be oxidized to nitrite which would accumulate as dry solid. Urea would hydrolyze to ammonia and would raise the pH to 7 to 8. AAOB are very active at this pH range and would lower the pH to about 6 where the NH₃ converts to ammonium and is unavailable.

Metabolism would be inhibited by low water activity as the scalp dried out. Under periods of intense sweating, the pores would be flooded with fresh sweat with a pH around 4 where decomposition of nitrite is significant and where AAOB can still metabolize urea into nitrite¹⁶⁰. This fresh sweat would dissolve accumulated nitrite and wick it toward regions of low pH due to the pH dependence of the surface tension of sweat (higher at low pH). The low pH regions are where AAOB are most active and are converting a cation (NH₄⁺) into an anion (NO₂⁻), lowering the pH. As the pores filled with sweat, the bottom of the biofilm would become anaerobic and the AAOB would use nitrite instead of O₂. Under anaerobic conditions (using gaseous NO₂ as well as nitrite) the consumption of NH₃, NO₂ and the production of NO go in the ratio of 1:2:1.¹⁶¹ Since the only exit route for nitrogen is as NO, essentially all NH₃ and urea excreted is converted to NO. Under these conditions, the average NO production from basal sweating would be about 125 μM/hr.¹⁶² Others have administered 1 mM NO/hr in inhalation air¹⁶³. If the pores of the biofilm fill with sweat, the diffusion resistance of a thickness of biofilm to nitric oxide could approach that of the skin. The skin thickness is limited by the diffusion resistance of nutrients from the capillaries to the living cells and so cannot become arbitrarily thick as the biofilm can.

These estimates have been incorporated into a one-dimensional model. The model is conservative in that no conversion of NO to GSNO is assumed between the source at the external skin surface and the 10 ppm boundary condition assumed on the interior and 10 micron thicknesses for the stratum corneum and 10 microns for the diffusive layer perfused by plasma. Actually, there would be continuous conversion of a portion of the NO to GSNO and SNO-albumin in that layer, so the "actual" diffusion resistance to NO would be less than what is assumed. NO also diffuses away from the skin through the biofilm, into the stagnant air on the scalp and is convected away into the ambient atmosphere. Some of this NO may be inhaled, but that part is neglected in this analysis. As an aside, facial hair may have evolved to supply NO for inhalation. Because women produce NO through activation of the estrogen receptor, their need for facial and body hair may be reduced compared to men. The permeability of the biofilm is not known, but for the purposes of this analysis several values are assumed (100, 10, 5 times greater and also the same diffusion resistance as the living portion of the skin ie. more permeable) and the results of the 1 dimensional model plotted in Figure 3 titled NO absorbed on Scalp. The total NO absorbed is plotted versus the thickness of the biofilm. Average NO₃ excretion for healthy individuals on low NO₃ diet is 70 $\mu\text{M}/\text{hr}$ ¹⁶⁴. NO from AAOB living on the scalp can be seen to produce absorbed levels comparable to normal basal NO production solely from normal sweat residues.

- This model assumes an ideal 2 dimensional surface with 1 dimensional diffusion. Actually, the skin is 3 dimensional, and these bacteria (some of which are motile) may migrate into the sweat ducts where they would have a better supply of urea, and where their NO would be absorbed better. The defining characteristic of mammals is the mammary gland, which is a modified sweat duct. All mammals have sweat glands, although many species do not use sweat glands for cooling, including rodents, dogs, and cats. Sweat glands are concentrated on the feet.
- Relying on bacteria to produce NO from the urea in naturally excreted sweat allows natural physiological mechanisms to regulate NO administration. Adrenergic mediated sweat on the scalp may occur for exactly that purpose.

Example

The inventor has had AAOB living on his unwashed skin for 15 months now (20 months on the scalp). During that time, his long term essential hypertension has resolved such that he no longer requires medication for its control, he has lost 30
5 pounds due to a decreased appetite, and without the discomfort that prior weight loss attempts have involved, and liver enzymes have declined into the normal range. He has experienced nocturnal priapism virtually every night. Subjectively, he has experienced greater mental acuity and greater tolerance for heat. Others have noted more vivid dream states.

10

Method of use of the present invention

According to an aspect of the invention, it is appreciated that many modern degenerative diseases may be caused by a lack of NO species, and that AAOB on the
15 external skin can supply those species by diffusion, and that application of AAOB to the skin resolves long standing medical conditions. In another embodiment of the invention, AAOB are applied to a subject to offset modern bathing practices, especially with anionic detergents remove AAOB from the external skin.

20 There are a number of different strains of AAOB. However, they are all very similar. They are all autotrophic, so none of them are capable of causing infection. The preferred strain would utilize urea as well as ammonia, so that hydrolysis of the urea in sweat would not be necessary prior to absorption and utilization by the bacteria. Also, in order to grow at low pH, the bacteria must either absorb NH₄⁺ ion, or urea.
25 The selected strain should also be capable of living on the external skin, and be tolerant of conditions there. The method I used to isolate such a strain, was to recover a mixed culture from barnyard soil, grow it in organic free media for some months, then apply it to my body, and some months later re-isolate the culture from my body. This selects for strains that are capable of living on the body.

30

The re-isolated culture is then grown in organic free media, and the active culture is then applied topically. One advantage of using organic free media is that there is no substrate for heterotrophic bacteria to metabolize except for that produced by the

autotrophic bacteria. Another advantage of using the as-grown culture is that substantial nitrite accumulates in the culture media, and this nitrite is also inhibitory of heterotrophic bacteria and so acts as a preservative during storage. When the active culture is applied, xanthine oxidase in the skin reduces the nitrite to nitric oxide, creating a "flush" of NO. While this prompt NO is important, the long term continuous administration of NO is more important.

The ideal method is to apply sufficient bacteria and then wear sufficient clothing so as to induce sweating. However, many people will want to derive the benefits of AAOB 10 while maintaining their current bathing habits, in which case, a culture of the bacteria can be applied along with sufficient substrate for them to produce NO. A nutrient solution approximating the inorganic composition of human sweat is optimal. Using bacteria adapted to media approximating human sweat minimizes the time for them to adapt when applied. Since sweat evaporates once excreted onto the skin surface, 15 using a culture media that has a higher ionic strength is desirable. The inventor has used a concentration approximately twice that of human sweat, but other conditions could work as well.

The AAOB have simple metabolic needs, NH₃ or urea, O₂, CO₂, and minerals. They 20 have a fairly high need for trace minerals including iron, copper, and zinc. Some strains also utilize cobalt, molybdenum, and manganese. They also need sodium, potassium, calcium, magnesium, chloride, phosphate and sulfate. All of these compounds are available in sweat in ratios not dissimilar to what is typically used in culture media for these bacteria.

25

Effects of AAOB on animal growth

According to another embodiment of the present invention, it is appreciated that 30 enhanced growth of cattle and the larger size, earlier puberty, and obesity of humans in industrialized areas are both due to the inhibition of the normal commensal AAOB. Accordingly, one aspect of the invention is an appreciation that animal growth may be augmented by the removal of AAOB. As used herein, the term "augment" is used to

define as an increase in weight, height, width, growth rate, and/or feed efficiency (weight gain per pound of feed). An interesting parallel can be made with animals that are raised for food. Many thousands of tons of antibiotics are incorporated into animal feed to increase growth rate and to increase feed efficiency. There is as yet, no good explanation of the mechanism by which antibiotics stimulate growth.¹⁶⁵ It has been suggested that they treat a "subclinical infection", or through the suppression of bacteria that would otherwise consume "nutrients", or by reducing nutrient consumption by the "immune system". These mechanisms seem implausible. A "subclinical infection" would be resolved by treatment, and continuous feeding of antibiotics would not be necessary. It would be surprising if every animal in a herd had the same "subclinical infection" and so each was helped to gain weight by the same amount. Similarly, is the immune system of every animal in a herd so over stimulated that they do not gain weight at an optimum rate? As for bacteria consuming nutrients, usually, animals are free to consume as much feed as they want. If bacterial consumption was a few percent higher, the animal could compensate by ingesting more, yet they do not. Also, antibiotic treatment does not render the digestive system of these animals bacteria free. On the contrary, populations of bacteria are still extremely high. Also, many bacteria develop resistance to these antibiotics and persist in spite of high levels.

The growth enhancing properties of antibiotics in feed may be mediated through inhibition of autotrophic ammonia oxidizing bacteria (AAOB) living on the external skin of these animals. In the wild, all animals which sweat (which includes all mammals) would be expected to have a population of ammonia oxidizing bacteria on their external skin metabolizing the urea in their sweat and producing NO and nitrite. Cattle are no exception. Giving large doses of antibiotics would be expected to result in antibiotics in the animals' sweat, and in the inhibition of any AAOB on the external skin. Inhibition of these bacteria would reduce basal NO levels, increase basal metabolism, increase growth rate, increase adult size, shorten the time to maturity, and increase body mass and body fat. These are exactly the changes that have been observed in human populations during industrialization. People get bigger, mature earlier, and become obese.

With this understanding, antibiotics in feed may not be necessary to inhibit AAOB on the external skin. A number of aspects of animal growth enhancement with antibiotics becomes understandable when it is recognized that AAOB are the target organism. AAOB have very small genomes. Nitrosomonas europaea has only 5 2,460¹⁶⁶ protein coding genes. It does not have genes for metabolizing xenobiotic compounds. It also does not have membrane transporters to excrete xenobiotic compounds. As an autotrophic bacterium it has a very slow metabolism, with a doubling time 30 times longer than that of heterotrophic bacteria. It would be expected to evolve 30 times slower, but since it also has such a limited genome, it 10 doesn't have the genes which can mutate and then perform new functions such as provide antibiotic resistance. Thus autotrophic bacteria would be expected to evolve antibiotic resistance much more slowly (if at all) than heterotrophic bacteria. AAOB are gram negative bacteria and are quite sensitive to many antibiotics.¹⁶⁷ Many of the antibiotics used in animal feed are not absorbed, but are excreted in the feces and 15 accumulate in manure. Manure contains abundant ammonia and urea and would in the absence of inhibitory compounds contain an abundance of AAOB. With antibiotics in animal manure, AAOB cannot grow, and so cannot inoculate the external skin of cattle. Using cattle as agents to mix antibiotics with manure and to apply it to their living areas would seem a less than ideal method. According to the 20 present invention, compounds to inhibit AAOB in the animal's living space could be applied directly.

AAOB are quite sensitive to compounds that inhibit the ammonia monooxygenase enzyme. Allylthiourea is such a compound that is very effective at inhibiting 25 ammonia monooxygenase and this compound is commonly used in waste water testing when determining biological oxygen demand, or BOD. Allylthiourea is added to inhibit the AAOB which would otherwise oxidize ammonia with oxygen and raise the measured O₂ consumption. Nitrification inhibitors are also used in fertilizer utilization. Many plants can absorb nitrogen both as ammonia and as nitrate. 30 However, for nitrogen to be incorporated into an amino acid, it must be in the ammonia form. Nitrate must therefore be reduced to ammonia. This reduction consumes energy that could otherwise be used to make plant biomass. It is therefore desirable in some instances to inhibit the nitrification bacteria in the soil when

nitrogen fertilizer is added in the form of ammonia or urea. A number of compounds are in common use in the fertilizer practice, and the use of any of these compounds would also be effective in blocking the nitrification of the urea in sweat when applied topically to the external surface of farm animals.

5

However, the safety of applying such compounds to animals is unknown. A better approach is to use an anionic detergent. AAOB are quite sensitive to anionic detergents, and are especially sensitive to linear alkylbenzene sulfonates (LAS) such as 4-(2-dodecyl)benzenesulfonic acid which has been shown to have a 50% inhibitory

10 concentration (IC₅₀) of 5, 3, 1, and 1 mg/L (ppm) for *N. europaea*, *N. mobilis*, *N. multiformis*, *Nitrosospira* sp. strain AV respectively. The critical micelle concentration (CMC) for LAS is 410 ppm, which is far above the IC₅₀ indicating a chemical effect rather than a detergency mediated effect.¹⁶⁸ They found that the AAOB tested did not develop resistance or tolerance when exposed to lower doses.

15 Although not bound by one particular theory, a possible reason anionic detergents are so toxic to the AAOB is that as anions, they are ported into the cell by the anion transporter which is necessary to bring in sulfate, phosphate and bicarbonate. Once inside, the AAOB doesn't have the metabolic machinery to get rid of it, either by metabolizing it into innocuous compounds, or to excrete it. Heterotrophic bacteria 20 easily adapt to high levels of LAS and many of them can utilize LAS as a carbon source. LAS is a common anionic detergent used in many cleaning products including dishwashing and laundry detergents though usually not shampoos because it is a little "harsh" and leaves the skin feeling "sticky". However, LAS is a high volume material with worldwide production (1987) of 1.8 million tons.¹⁶⁹ Huge

25 quantities are already discharged into the environment, so using it to inhibit AAOB on the skin of farm animals would not be expected to have any environmental impact. In any case, using LAS for farm animal growth enhancement would displace the antibiotics which are already being used and which are already a far worse problem due to induction of antibiotic resistance in pathogenic bacteria. There is extensive 30 data on the safety and irritancy of LAS, but most studies do not look at concentrations far below the CMC, likely because the effects there are so small. In practice, the detergent solution could be sprayed on the animal, and then not rinsed off, or the animal would be forced to swim through a bath of the material. The detergency of a

surfactant is approximately constant above the CMC, and approximately linear with concentration below the CMC. Most of the adverse effects of detergents on the skin are due to protein denaturing and defatting of the skin. Because detergency is not required for inhibition of AAOB, levels that denature proteins and defat the skin are not required. One way to ensure a long term inhibitory dosage on the skin is to form a low solubility "soap" in situ. A solution of LAS in water is sprayed on the animal, and then a solution of a divalent salt, such as calcium chloride is sprayed on as well. Mixing would occur on the skin, where the LAS would precipitate as the relatively insoluble calcium LAS soap. The precipitated soap would adhere to the animals hair and so provide a reservoir of LAS which would dissolve as the animal sweated or was rained upon. The amount of precipitated LAS could be adjusted to attain an inhibitory level of LAS between treatments. The solubility product K_{sp} for LAS (carbon number ~12, average MW=343) is 8.4e-12.¹⁷⁰ The calcium content of human sweat is 3 mM/L. Assuming a similar value, for cattle sweat, then at the solubility limit of Ca(LAS)₂, the LAS concentration would be 18 ppm. This is sufficiently high that AAOB would be substantially inhibited so long as there was any residual Ca(LAS)₂ soap present on the cattle. The initial concentration would be much higher when the detergent is first sprayed on. Other molecular weight LAS compounds have different K_{sp}'s. For example, an LAS with a MW of 339 (carbon number ~11.4) has a K_{sp} of 1.8 e-11. This represents a concentration of 26 ppm.

No doubt other inhibitors can be used, but there are few materials as cheap and as benign and as readily available as LAS.

25 What is claimed is:

58
CLAIMS

1. A method of treating a subject who has developed or is at risk of developing at least one of hypertension, hypertrophic organ degeneration, Raynaud's phenomena,
5 fibrotic organ degeneration, allergies, autoimmune sensitization, end stage renal disease, obesity, impotence and cancer comprising:
positioning ammonia oxidizing bacteria in close proximity to the subject.

- 10 2. The method of claim 1, wherein the act of positioning the bacteria comprises positioning a bacteria selected from the group consisting of any of *Nitrosomonas*, *Nitrosococcus*, *Nitrosospira*, *Nitrosocystis*, *Nitrosolobus*, *Nitrosovibrio*, and combinations thereof.

- 15 3. A method of retarding the progression of aging of a subject comprising:
positioning ammonia oxidizing bacteria in close proximity to the subject.

4. A method of augmenting animal growth comprising:
removing AAOB from the surface of the animal.

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Fig 1

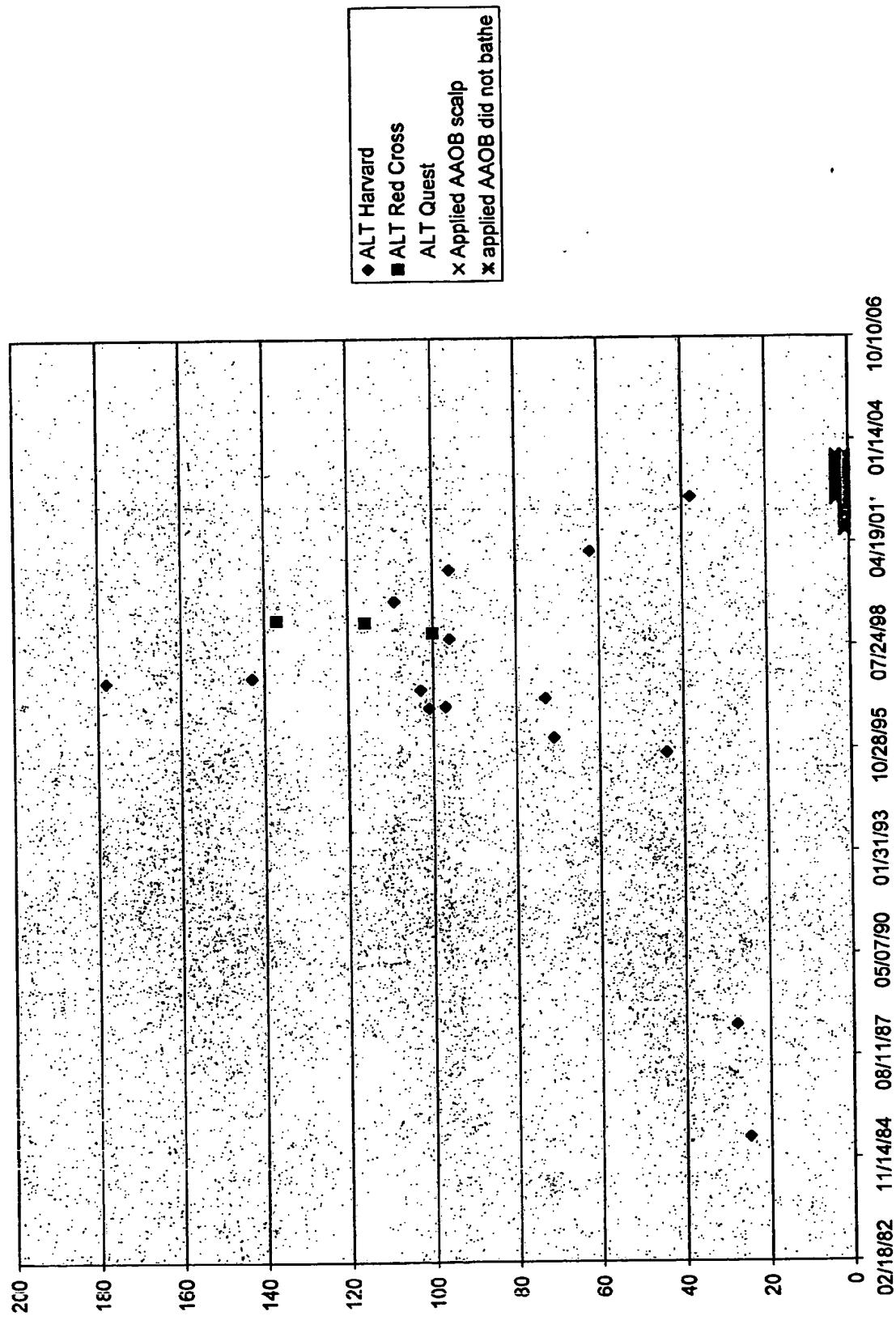


Fig 2

F. Alzheimer's Disease verses Temperature

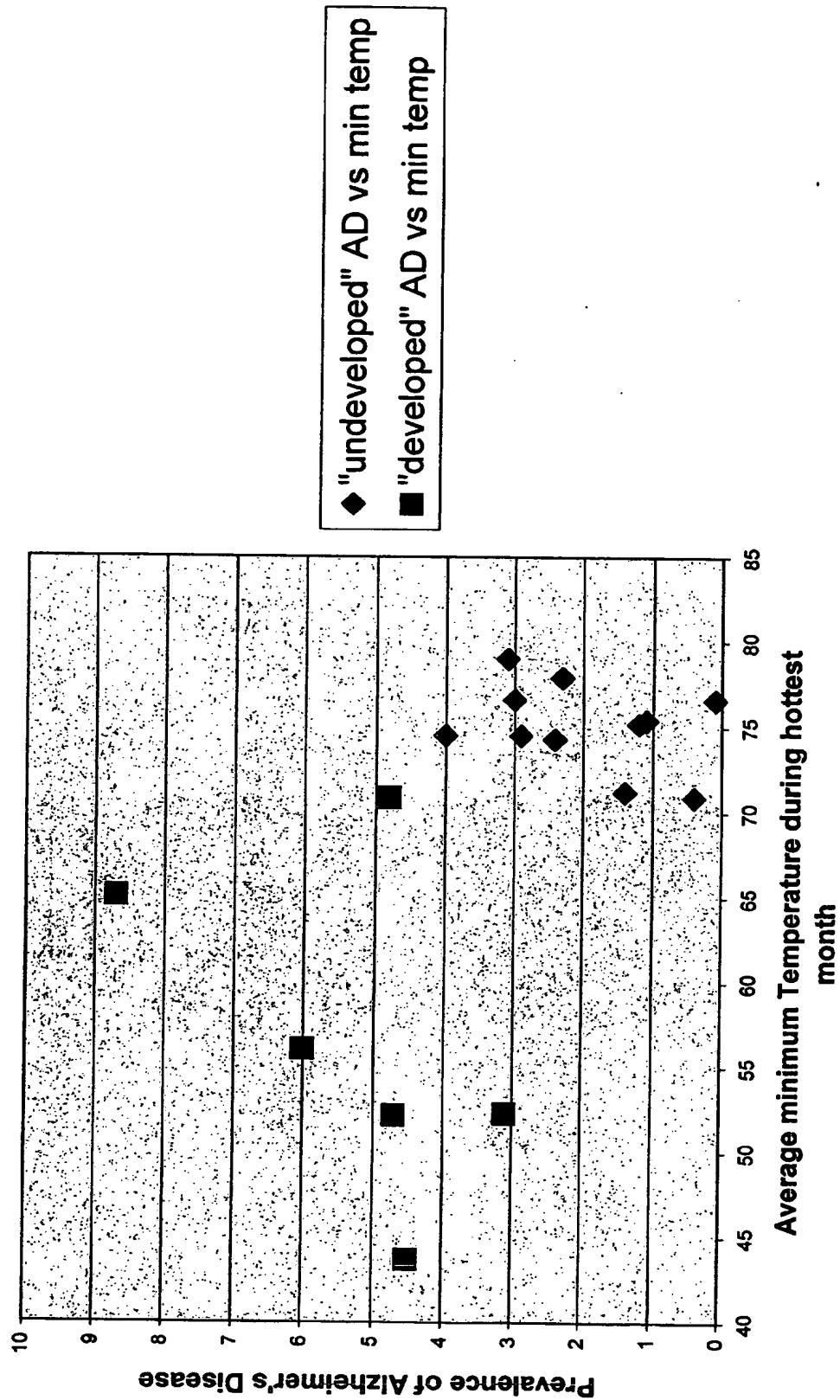


Fig 3

Figure 4. Number of US Patents with 510/119 classification

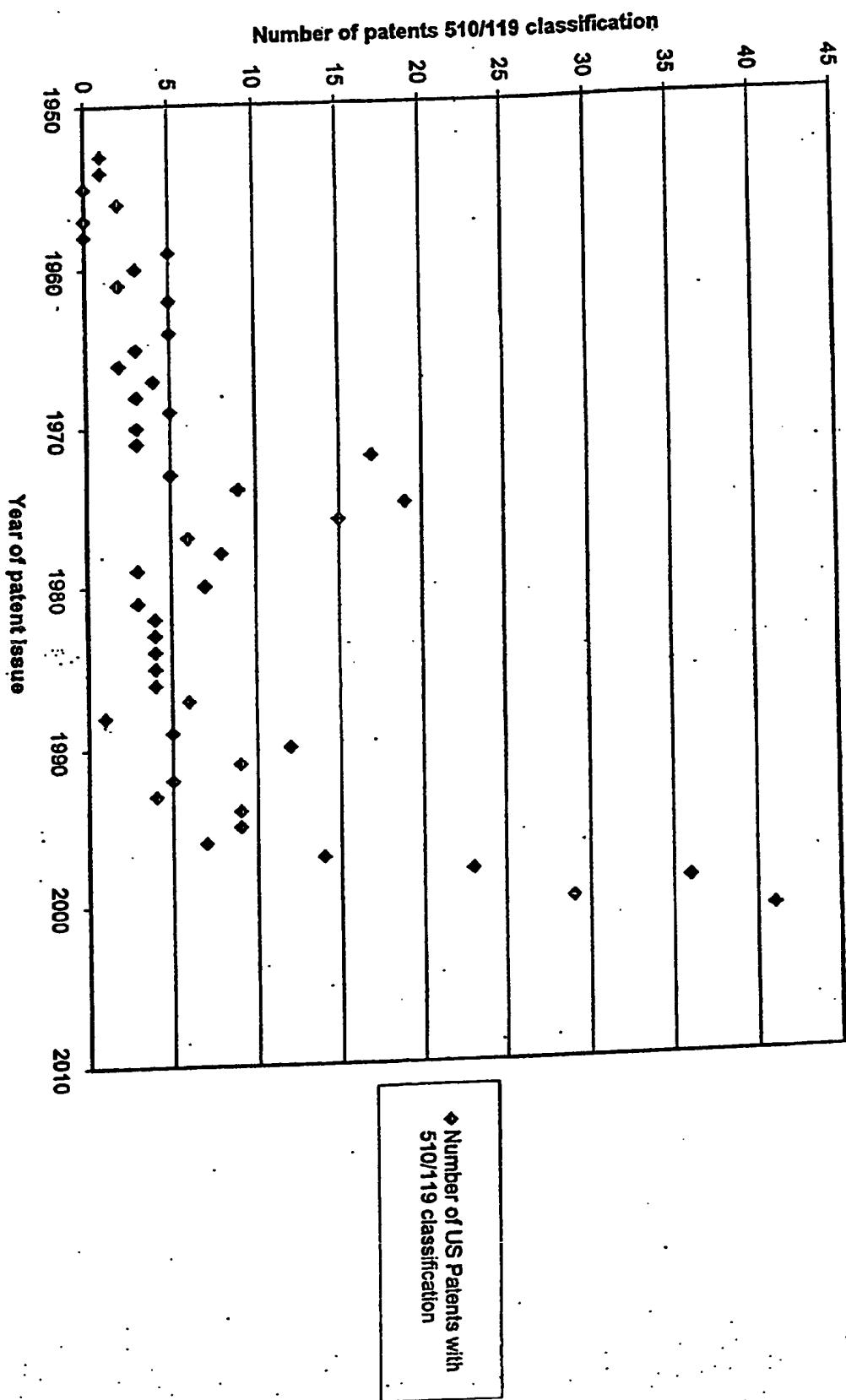
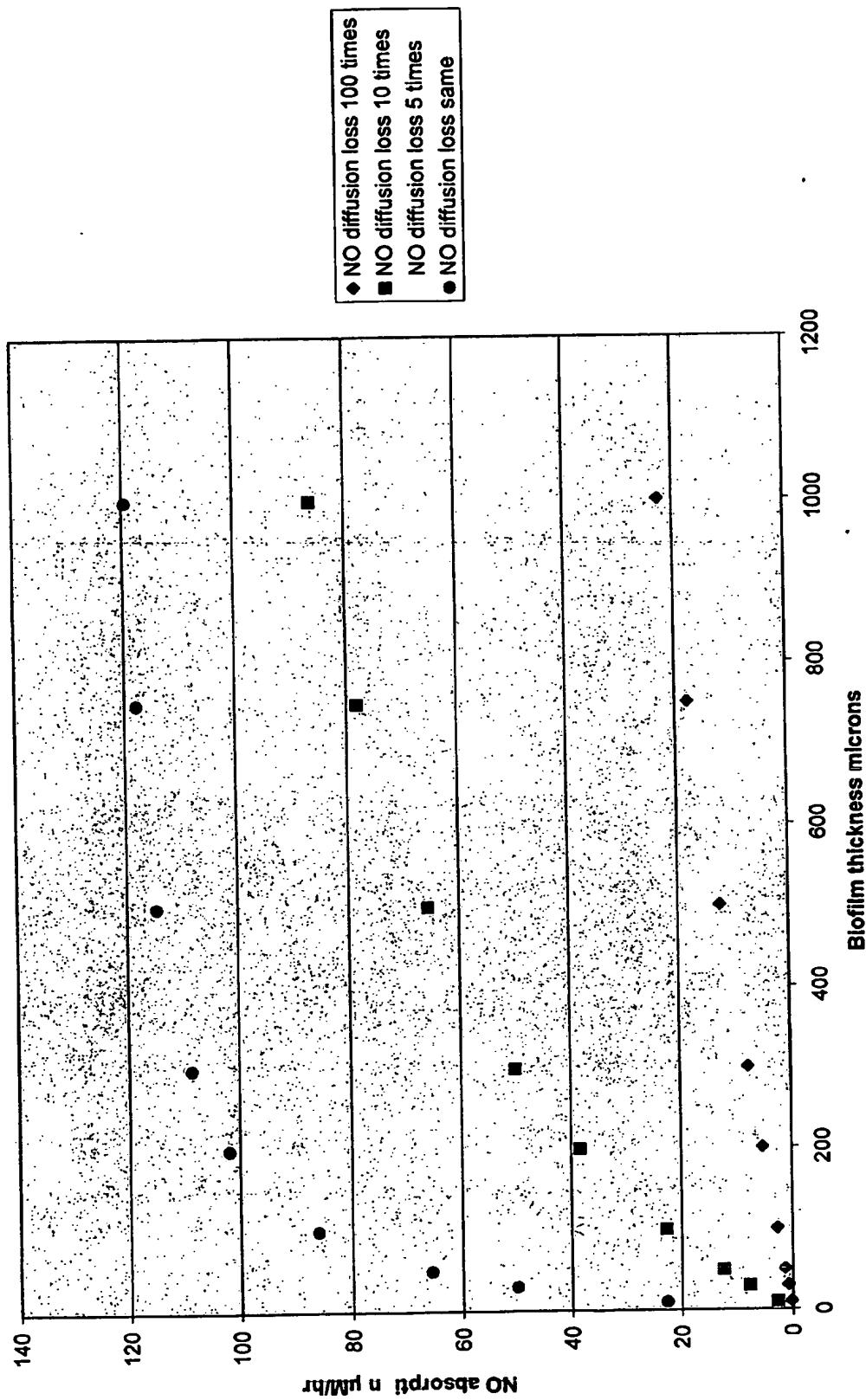


Fig 4

NO absorbed scalp from basal sweat



APPLICATION DATA SHEET

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Subject Matter:: Utility
Title:: METHODS OF USING AMMONIA OXIDIZING
BACTERIA
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